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Functional and Genetic Identification of Lineage Committed Intrathymic Progenitors in Adult Mice

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Abstract

T-lymphocytes are an essential component of the adaptive immune system and require a distinct anatomical location for their development, namely the thymus. This study focuses on early events during development of these cells in the adult mouse.

The most immature compartment of murine thymocytes is subdivided by the cell surface expression of CD44 and CD25. The most primitive progenitor population resides in the CD44+CD25– double negative 1 (DN1) fraction and can be further subdivided according to expression of the receptor tyrosine kinase c-kit (CD117). The DN1 CD117+ fraction is homogeneous, unlike the CD117– fraction, which constitutes a preponderant CD45R+CD127– population and a smaller CD45R–CD127+ population.

Analysis of gene expression was employed to understand the relationship between the DN1 CD117+ (DN1 CD117) and the DN1 CD45R+ (DN1 CD45R) populations. Expression of genes associated with the T-cell lineage, such as Notch-1, Runx-1 and Rag-1 was observed in both populations. Furthermore, substantial expression levels of genes involved in T-cell commitment, such as pre-Tα, in the DN1 CD45R population could reflect the initiation of T-cell fate specification.

In parallel, a *Cre* recombinase based lineage tracing approach was utilised to further dissect the earliest thymocyte pools. Upon expression of the *Cre* recombinase, all cells and their progeny are permanently marked by the expression of a fluorescent reporter protein. In this study, the human CD2 (hCD2) promoter and locus control regions drove expression of the *Cre* recombinase. Analysis of the DN1 population revealed that nearly all the DN1 CD117 fraction was reporter negative, whereas the DN1 CD45R subset was completely reporter positive. Since expression of hCD2 has so far been reported only in the lymphoid lineage, this observation further underlines the fundamental differences between the two progenitor fractions and indicates their alternate developmental origins.

To address the developmental potential of these populations, the DN1 CD117 and the DN1 CD45R pools were functionally assessed *in vitro* and *in vivo*. The DN1 CD117 population showed a robust T-, B- and NK-cell potential *in vitro* and *in vivo*, in addition to a residual myeloid activity *in vivo*, whereas the DN1 CD45R fraction was strongly biased towards a CD8 NK-T cell lineage *in vivo*.

In summary, these data indicate that developmentally distinct progenitor populations seed the adult murine thymus and can potentially contribute to discrete subsets of mature haematopoietic cells.

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Abbreviations

ALK-2	activin receptor like kinase 2
APC	allophycocyanin
Arbp	acidic ribosomal phosphoprotein
BMP7	bone morphogenic protein 7
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
c-kit	proto-oncogene tyrosine protein kinase Kit
CDC42	cell division cycle 42
CLP	common lymphoid progenitor
CLP-2	common lymphoid progenitor 2
CMP	common myeloid progenitor
COI	centre of intensity
cRNA	complementary ribonucleic acid
CSF1R	colony stimulating factor 1 receptor
DN	double negative
DOCK2	dedicator of cytokinesis 2
DP	double positive
ECM	extracellular matrix
EGFP	enhanced green fluorescent protein
ELP	early lymphoid progenitor
EpoR	erythropoietin receptor
ESC	embryonic stem cell
ETP	early thymic progenitor
EYFP	enhanced yellow fluorescent protein

FACS	fluorescence activated cell sorter
FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
Flk-2	foetal liver kinase 2
Flt-3	fms-related tyrosine kinase 3
Flt-3L	fms-related tyrosine kinase 3 ligand
FTOC	foetal thymic organ culture
GCOS	genechip operating software
G-CSFR	granulocyte colony stimulating factor receptor
GMP	granulocyte/monocyte progenitor
hCD2	human CD2
HSC	haematopoietic stem cell
ICAM	intercellular adhesion molecule
ICN	intracellular notch
iCre	improved Cre recombinase
IL-7	interleukin 7
IL-7R	interleukin 7 receptor
Lin-	lineage negative
LMPP	lymphoid primed multipotent progenitor
LSK	lineage negative Sca-1 positive c-kit positive
Ly-6A/E	lymphocyte antigen 6 complex locus A/E
Ly-6G	lymphocyte antigen 6 complex locus G
MEP	megakaryocyte/erythroid progenitor
MFI	mean fluorescence intensity
MM	mismatch

MMP	matrix metalloproteinase
MPP	multipotent progenitor
NK	natural killer
NSC	neural stem cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGFR	platelet derived growth factor receptor
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PerCP-Cy5.5	peridinin chlorophyll protein cyanine 5.5 tandem dye
PI3K	phosphatidylinositol 3 kinase
PLCγ	phospholipase C gamma
PM	perfect match
Ptk2	protein tyrosine kinase 2
R26	Rosa26 locus
RAG-1	recombinase activating gene 1
RAG-2	recombinase activating gene 2
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
Sca-1	stem cell antigen 1
SCF	stem cell factor
SP	single positive
TCR	T cell receptor
TSP	thymic seeding progenitor
VCAM	vascular cell adhesion molecule

Chapter 1

Introduction

The Thymus

The ancient Greeks, whilst performing sacrificial rituals on young animals, noted a large mass of tissue located in the chest above the heart protruding for some distance up into the neck and concluded it to be the "seat of the soul". This organ was named the thymus, which vaguely translates as soul, heart, courage, mind, will or purpose. As early as 1777, William Hewson noted that the thymus was filled with 'particles' resembling those in blood and lymph and by the second part of the 20th century the immunological relevance of the thymus was unequivocally recognised. Work carried out in the 1960s by Jacques Miller and colleagues illustrated that neonatally thymectomised mice showed a marked deficiency of small lymphocytes in spleen, lymph nodes, Peyer's patches and peripheral blood. In addition, these animals were unable to elicit cell mediated as well as humoral immune responses (Miller 1962). The immunological competence of these animals was re-established upon them receiving a thymus graft (Miller 1962). This prompted Miller to conclude that the thymus was responsible for the development of immunologically competent cells. At this point the nature of cells produced by the mammalian thymus was still unclear, unlike that of the chicken. Burnet and colleagues demonstrated that there is a division of labour between lymphocytes in the chicken (Warner, Szenberg 1962): those produced by the bursa of Fabricius were responsible for the production of antibodies and delayed-type hypersensitivity, whereas cells arising from the thymus were accountable for cellular immunity (Warner, Szenberg 1962). Since the work of Miller showed that both branches of the adaptive immune system were impaired upon neonatal thymectomy, it was assumed that the mammalian thymus performed the dual role of cell-mediated and humoral immunity. However, this was questioned by the observation that neonatally thymectomised mice had a deficiency of lymphocytes restricted to areas of lymph nodes

and spleen associated with histological changes induced by cell-mediated immune responses but not in those areas where antibody producing cells appeared (Parrot, de Sousa et al. 1966). Subsequent experiments by Mitchell and Miller mapped the origin of the antibody forming cells to the bone marrow, which were later called B-cells, and the thymus derived cells, termed T-cells, were essential in assisting B-cells to produce antibody as well as being responsible for cellular immunity (Mitchell and Miller 1968).

The thymus can be divided into two histologically defined regions, the cortex and the medulla, each containing several different types of thymic epithelial cells (as reviewed by Blackburn and Manley 2004). During embryonic development in mice, the thymus originates from the third pharyngeal pouch endoderm between embryonic day 10 and 11. Subsequently, the primordia separate from the pharynx and begin migration towards the anterior chest cavity. By day 14 of embryonic gestation the thymus can be identified as a distinct organ occupying the approximate adult position in the embryo (Blackburn, Manley 2004; Anderson, Jenkinson et al. 2006).

Early studies proposed that lymphocytes inhabiting the thymus originate from the epithelial component of the organ (Auerbach 1960; Auerbach 1961). However, studies with interspecific chimeras clearly demonstrated an extrinsic origin of thymus-derived lymphocytes (Le Douarin, Jotereau 1975). Transplantation of the total thymic anlage from quail embryo into the somatopleure of a 3 day old chick host revealed that the implanted organ was of the quail origin, whereas the lymphocytes residing in it were exclusively derived from the host, which lead to the conclusion that the thymic epithelia does not have any ability to differentiate into lymphocytes (Le Douarin, Jotereau 1975). Instead, lymphocytes developing in the thymus require an input from an alternative source. In the adult, this is the bone marrow.

Haematopoiesis

The process of formation of all blood cell lineages is termed haematopoiesis. Haematopoiesis originates from a very rare population of haematopoietic stem cells (HSC) in the bone marrow of an adult individual. Bone marrow HSCs share the classic functional properties of all stem cells, namely self-renewal and pluripotency, in addition HSCs have unique radioprotective properties. These functional characteristics were later supplemented with a phenotypic definition and allowed the isolation of the radioprotective entity by fluorescently activated cells sorting (FACS). It is now established that a single HSC of the $Lin^{-}CD117^{+}Ly6A/E^{+}CD90^{low}$ surface phenotype can give rise to long-term multilineage reconstitution and self-renewal in irradiated mice. It has also been demonstrated that bone marrow harbours unexpected potential to generate non-haematopoietic lineages. These include a robust potential to generate hepatic cells and a less robust potential to produce skeletal muscle, cardiac muscle and neural cells.

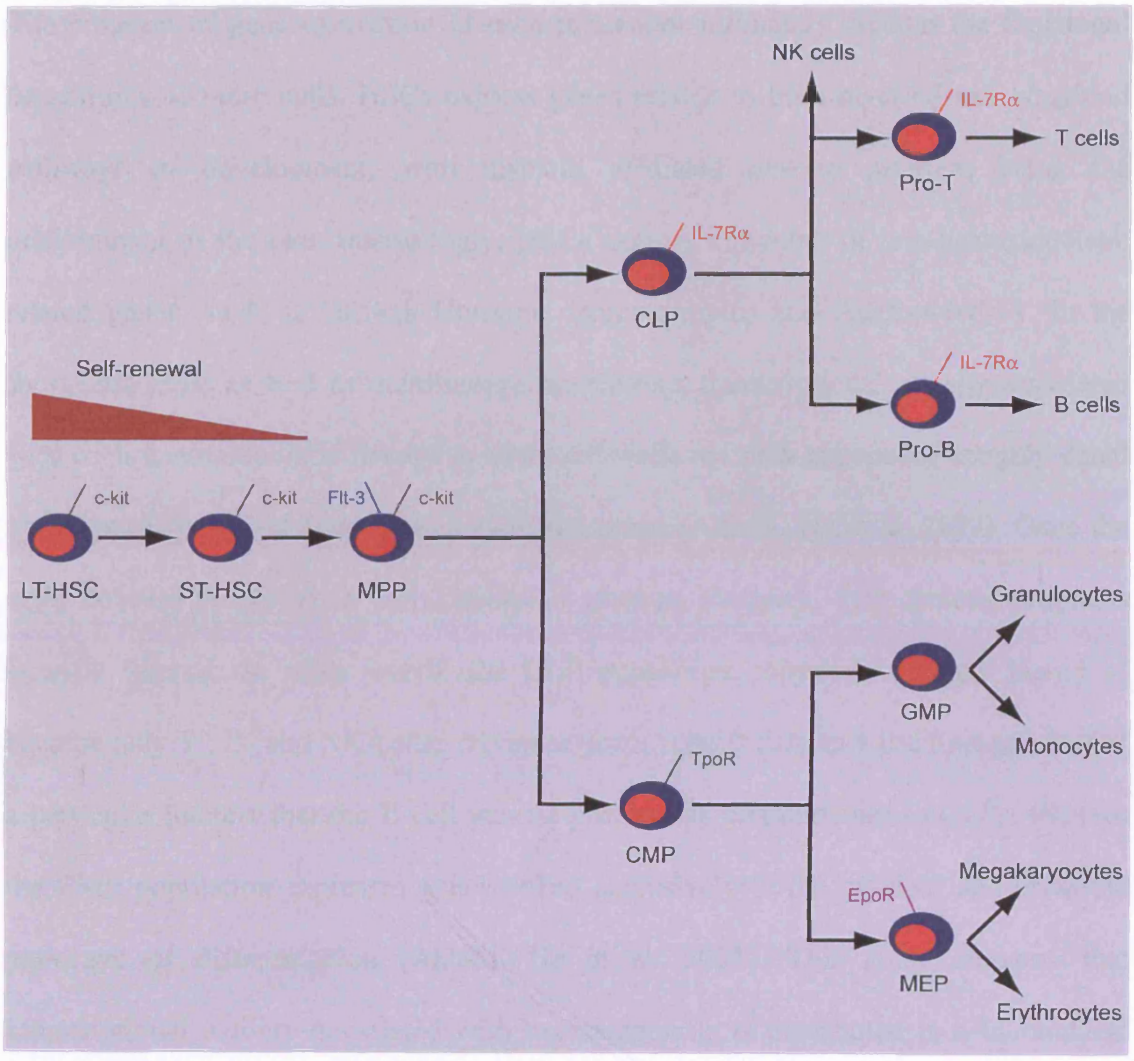
The prevailing model of haematopoiesis as proposed by I. L. Weissman and colleagues is illustrated in Figure 1.1.

A long-term HSC gives rise to a short-term HSC, the two populations can be clearly distinguished phenotypically by the expression of CD34 on the short-term HSC (Osawa, Hanada et al. 1996). These cells retain multilineage capacity but their self-renewal capabilities are diminished. In turn, short-term HSC gives rise to the multipotent progenitor (MPP) population, which is marked by expression of foetal liver kinase-2 (flk-2, flt-3, CD135) (Adolfsson, Borge et al. 2001). As the name suggests, the MPP possesses a multipotent functional potential, however lacking any self-renewal competence. According to this model the primary step towards lineage commitment is a strict separation between the lymphoid and the myeloid pathways of haematopoietic

development, where the MPPs give rise to lineage restricted progenitors, with the cell surface expression of CD127 (interleukin 7 receptor alpha chain, IL-7Rα) marking the earliest lymphoid pool - the common lymphoid progenitor (CLP) (Kondo, Weissman et al. 1997). Correspondingly, a CMP represents a common myeloid progenitor for both granulocyte/monocyte (GM) and megakaryocyte/erythroid (Mk/E) pathways (Akashi, Traver et al. 2000) (Figure 1.1).

Figure 1.1 A Prevailing Model of Haematopoiesis.

A model of haematopoietic development as proposed by Weissman I.L. Generation of all blood cell lineages originates from a population of haematopoietic stem cells (HSC) which are capable of self-renewal. Progression to the multipotent progenitor stage is accompanied by the loss of self-renewal ability and upregulation of flt-3 (flk-2, CD135). This model proposes a bifurcation at this point separating the lymphoid and the myeloid stages of differentiation. Lymphoid commitment is marked by upregulation of the interleukin-7 receptor α chain (CD127) and generation of the common lymphoid progenitor (CLP), whereas myeloid differentiation is accompanied by cell surface expression of the thrombopoietin receptor (CD110) and consequent generation of the common myeloid progenitor (CMP). The CLP gives rise exclusively to cells of the lymphoid lineage, whereas the CMP is restricted to generation of myeloid lineage cells only.


NK cells
IL-7Rα
T cells
Pro-T
IL-7Rα
CLP
Self-renewal
IL-7Rα
B cells
Pro-B
c-kit
c-kit
Flt-3
c-kit
LT-HSC
ST-HSC
MPP
Granulocytes
GMP
Monocytes
TpoR
CMP
Megakaryocytes
EpoR
MEP
Erythrocytes

The program of gene expression of each progenitor ultimately dictates the functional capabilities of these cells. HSCs express genes related to both myeloid and lymphoid pathways of development, with myeloid affiliated genetic program being the predominant of the two. Interestingly, HSCs express a number of non-haematopoietic related genes, such as Growth Hormone (somatotropin) and Angiopoietin-1. In the short-term HSC as well as multilineage progenitors, transcriptional activity associated with each haematopoietic lineage is equalised, cells are now expressing roughly equal numbers of lymphoid and myeloid affiliated genes (Akashi, He et al. 2003). Once the cells become restricted in their ability to generate progeny, their genetic programs become filtered. In other words, the CLP population, which is strongly biased to become only T-, B- and NK-cells, expresses genes related only to those lineages. It is of a particular interest that the B-cell genetic program is preponderant in CLPs, whereas the CMP population expresses genes linked exclusively to the myeloid and erythroid pathways of differentiation (Akashi, He et al. 2003). This study suggests that transcriptional activity associated with haematopoiesis is distributed in a hierarchical and asymmetrical fashion with expression of lineage affiliated genes preceding final commitment at the cellular level. Expression of different lineage specific genetic programs might reflect lineage potentials, whereas refinement of gene expression might correlate with the cells commitment to terminal differentiation.

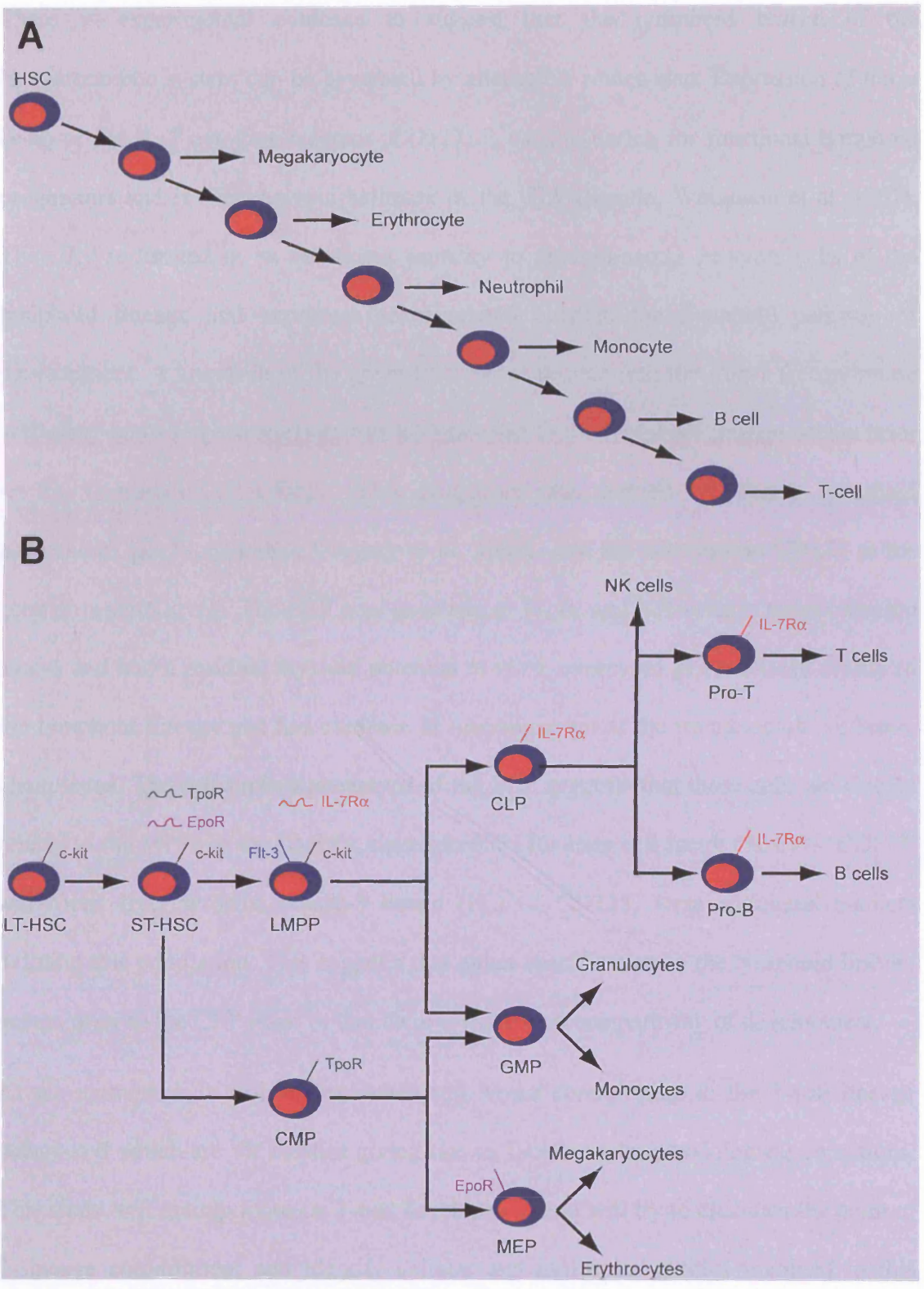
A recent report identifying a B-macrophage progenitor in the adult bone marrow is questioning the idea of an early dichotomy between myeloid and lymphoid lineages during haemato-lymphoid development. This population bears cell surface markers associated with lymphoid cells – CD127 (IL-7Rα), CD93 (AA4.1), CD19, generates only B-cells and macrophages at the clonal level and expresses genes related to both lineages (Montecino-Rodriguez, Leathers et al. 2001).

An alternative model has been suggested by Brown in 1985 and is illustrated in Figure 1.2A. This model advocates a sequential loss of functional capacity by progenitor cells as they progress along the developmental axis. Some recent experimental evidence lends support for this scheme (Adolfsson, Mansson et al. 2005). These data demonstrate the loss of megakaryocyte/erythroid potential as an initial step in blood cell differentiation. The long-term HSC gives rise to the short-term HSC, which in turn generates a lymphoid primed multipotent progenitor (LMPP). Phenotypically the LMPP population resembles the MPP population, both fractions retain the capacity to produce lymphoid as well as myeloid progeny, however, the LMPP lacks any ability to manufacture cells of the megakaryocyte/erythroid lineages. The functional attributes of LMPPs are reflected in the genetic program of these cells. There is complete absence of gene expression of elements known to be essential for erythroid development, such as GATA-1 and erythropoietin receptor (EpoR), or megakaryocyte development, such as the thrombopoietin receptor (TpoR). On the other hand, there is marked upregulation of CD127 and sustained expression of granulocyte-colony stimulating factor receptor (G-CSFR, CD114), signalling through which supports myeloid development. A further restriction in functional capabilities of the LMPP is mirrored in the loss of granulocyte/monocyte potential thus yielding a common lymphoid progenitor. These authors offer their own view on haematopoietic development (Figure 1.2B), which implies that granulocyte/monocyte cells can be generated by two distinct pathways, one via the CMP and one via the LMPP.

Figure 1.2 Alternative Models of Haematopoiesis.

A A model of haematopoietic development as proposed by Geoffrey Brown. Development of all blood cell lineages originates from a haematopoietic stem cell (HSC). This model proposes that there is a predetermined order of developmental outcomes, at any one point a progenitor cell has at most two developmental choices.

B A composite model proposed by Jacobsen hypothesises that lineage determination is achieved by gradual loss of alternative cell fates, in this manner, the erythroid lineage is lost first, thus generating a lymphoid primed multipotent progenitor, which harbours a potential to generate all blood cell lineages excluding erythrocytes, but biasing the lymphoid lineage. This is reflected in upregulation of genetic elements associated with the lymphoid pathway of differentiation. Additionally, this model proposes that a single haematopoietic lineage can be derived via distinct intermediates.


A
HSC
Megakaryocyte
Erythrocyte
Neutrophil
Monocyte
B cell
T-cell
B
NK cells
IL-7Rα
T cells
Pro-T
IL-7Rα
CLP
TpoR
EpoR
IL-7Rα
c-kit
c-kit
Flt-3
c-kit
IL-7Rα
B cells
Pro-B
LT-HSC
ST-HSC
LMPP
Granulocytes
GMP
Monocytes
TpoR
CMP
Megakaryocytes
EpoR
MEP
Erythrocytes

There is experimental evidence to suggest that the lymphoid branch of the haematopoietic system can be generated by alternative routes also. Expression of the α chain of the IL-7 cytokine receptor (CD127) is used to enrich for functional lymphoid progenitors and is a phenotypic hallmark of the CLP (Kondo, Weissman et al. 1997). The CLP is limited in its functional capacity to predominantly generate cells of the lymphoid lineage and expresses genes related only to the lymphoid pathway of development. A knock-in of the green fluorescent protein into the *Rag-1* (recombinase activating gene-1) gene suggests that commitment to the lymphoid lineage occurs prior to the expression of CD127. This progenitor was termed the "early lymphoid progenitor" (ELP) (Igarashi, Gregory et al. 2002), and did not express CD127 at the gene or protein levels. The ELP pool gave rise to T-, B- and NK-cells in transplantation assays and had a residual myeloid potential *in vitro*, expressed genes related mostly to the lymphoid lineage and had evidence of rearrangement at the immunoglobulin heavy chain locus. The cell surface phenotype of the ELP suggests that these cells are closely related to the MPPs or the LMPPs, since receptors for stem cell factor (SCF) — CD117, and foetal liver tyrosine kinase-3 ligand (FL) — CD135, were additional markers defining this population. This suggests that either specification to the lymphoid lineage occurs prior to the CLP stage or that there is more than one pathway of development.

At the moment it is still unclear when and where commitment to the T-cell lineage occurs and which are the entities giving rise to T-cells under physiological conditions. This study will mainly focus on T-cell development and will try to elucidate the point of T-lineage commitment and identify cellular and molecular entities involved in this process.

T-cell Development

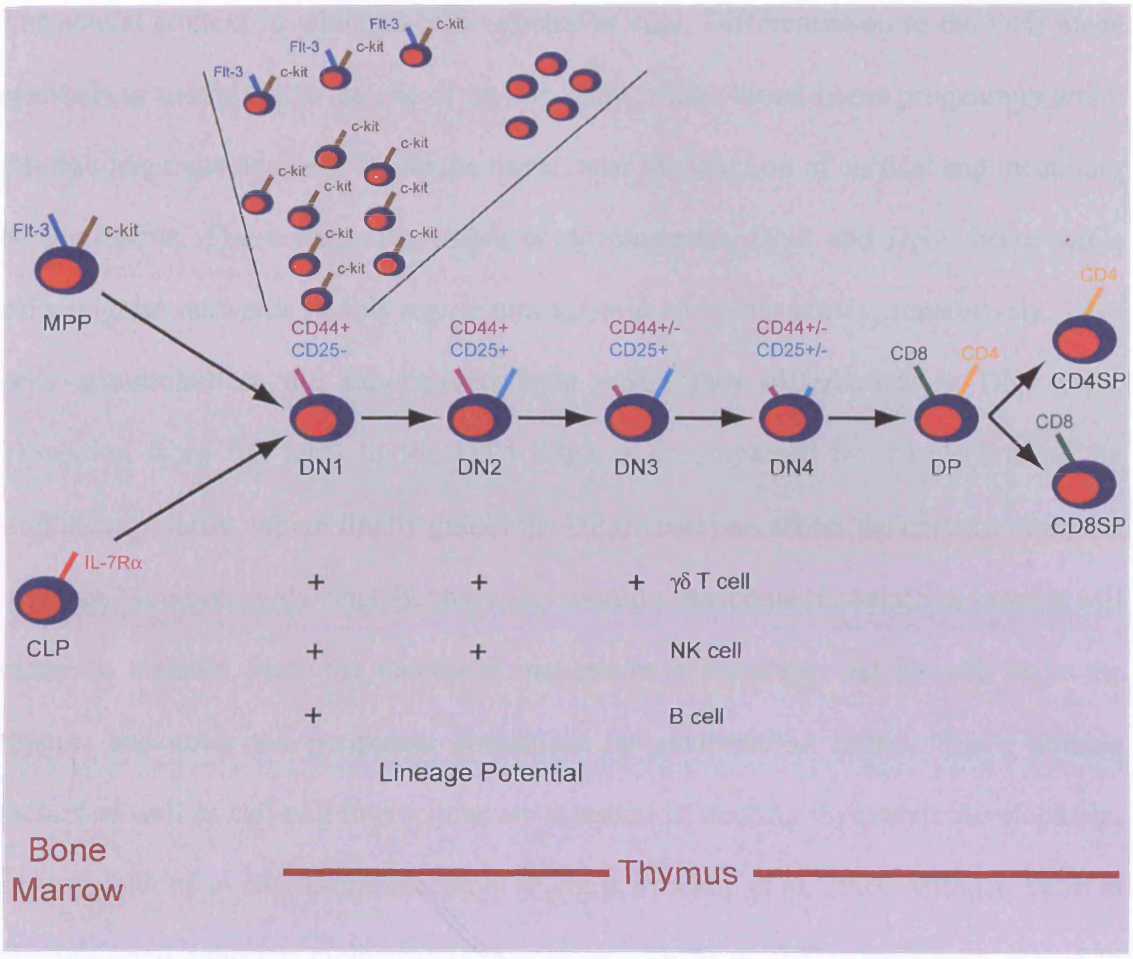
The requirement of a specialised anatomical site for T-cell development is exemplified by mutations in the forkhead transcription factor *FoxN1* gene, which renders these animals athymic and without any noticeable T-cell repertoire (Nehls, Pfeifer et al. 1994). Since the thymus has no capacity to generate T-cell precursors and solely relies on the input from the bone marrow, a particular cell has to leave the bone marrow and travel to the thymus. After homing to the thymus, cells undergo a stepwise and ordered process of development marked by the expression of two co-receptors, namely CD4 and CD8 (Ceredig, Dialynas et al. 1983) (see Figure 1.3). The earliest population expresses neither molecule and is therefore called the double negative (DN) population. Upon maturation these cells acquire expression of both CD4 and CD8 and thus progress to the double positive (DP) stage of development. At this point the differentiation path forks and cells become either $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ single positive (SP) cells. The transition from a DP to a SP thymocyte is usually the outcome of a complex biological process, known as selection, which eliminates any harmful autoimmune, self reactive potential (negative selection) and the concomitant accumulation of "useful" T-cell clones (positive selection).

The DN population is the smallest population in the thymus and contains the recent thymic immigrants. It can be subdivided into four distinct subsets according to their expression of CD44 and CD25 (Lesley, Hyman et al. 1985; Ceredig, Lowenthal et al. 1985). The most primitive population, the DN1 population, expresses only CD44 on its surface. Once CD25 is upregulated cells proceed to the next developmental stage, the DN2 stage, upon downregulation of CD44 cells become DN3 cells and finally loss of CD25 defines the final step of DN development, the DN4 stage (see Figure 1.3).

Figure 1.3 T-cell Development in the Thymus.

T-cell development in the thymus is defined by the cell surface expression of CD4 and CD8. The most primitive progenitors do not express CD4 or CD8 and are therefore called double negative cells. These in turn can be separated based on the surface expression of CD44 and CD25. The most primitive progenitor population in the thymus expresses CD44 but not CD25 and is called double negative 1 (DN1). These cells are heterogeneous and contain cells which express the receptor tyrosine kinase c-kit (CD117). These have been shown to harbour the most robust T-cell differentiation potential and have been called the early thymic progenitor (ETP). A fraction of DN1 CD117 cells express flt-3 (CD135) and by this cell surface phenotype resemble multipotent progenitor cells in the bone marrow. It is thought that flt-3 positive DN1 CD117 positive cells constitute the thymic seeding progenitors (TSP), the earliest population in the adult thymus, and it is this population that generates the rest of the DN1 CD117 positive pool. An additional population exists in the DN1 fraction, which does not express CD117, and it is believed that these cells do not contribute to T-cell ontogenesis.

Functionally, the DN1 population is able to generate a number of haematopoietic lineages, which include all T-cells, B-cells and natural killer (NK) cells. Upon subsequent differentiation the ability to generate B-cells is lost, and at the DN3 stage the cells are destined to form only T-cells.


Flt-3
c-kit
MPP
CD44+
CD25-
CD44+
CD25+
CD44+/-
CD25+
CD44+/-
CD25+/-
CD8
CD4
CD4SP
CD8
CD8SP
DN1
DN2
DN3
DN4
DP
IL-7Rα
CLP
+
+
+
γδ T cell
+
+
NK cell
+
B cell
Lineage Potential
Bone
Marrow
Thymus

The spatial context in which T-cells develop is vital. Differentiation to the DN1 stage proceeds in proximity to the site of thymic entry, where blood-borne progenitors arrive through large venules deep inside the tissue, near the junction of cortical and medullary compartments. The consecutive stages of development, DN2 and DN3, occur while cells migrate outwards of this region into the mid and outer cortex, respectively. DN3 cells accumulate in the sub-capsular zone where they differentiate to DN4 cells. Transition from the DN3 to the DN4 stage is accompanied by a reversion of the migration polarity, which finally guides the DP thymocytes across the cortex toward the medulla. However, only cells that have successfully overcome the selection process will enter the medulla. Here the functional maturation is complete, and SP cells leave the thymus and enter the peripheral circulation (as reviewed in Petrie, 2003). Soluble factors as well as cell-cell interactions are essential in steering thymocyte development, since mutations in chemokine receptors (Plotkin, Prockop et al. 2003; Misslitz, Pabst et al, 2004) and adhesion molecules (Schmeissner, Xie et al. 2001) have been shown to affect T-cell development.

The DN1 population of cells harbours the potential to generate cells of various lineages, such as B-cells, dendritic cells and NK-cells as assessed by *in vivo* transplantation experiments, and even a residual myeloid potential in an *in vitro* culture system (Wu, Antica et al. 1991; Ardavin, Wu et al. 1993; Lee, Kim et al. 2001; Balciunaite, Ceredig et al. 2005). The ability to generate myeloid, B- and dendritic cells is diminished once cells mature to the DN2 stage whilst still retaining the NK- and T-cell potentials (Wu, Antica et al. 1991). The rearrangement of the β chain of the TCR is initiated at this point and completed by the DN3 stage. DN3 cells loose the capacity to develop along the NK lineage and can only form α/β or γ/δ T-cells, γ/δ T-cell precursors being marked by cell surface expression of CD27 (Taghon, Yui et al. 2006). Successful rearrangement of the

β chain results in the assembly of a pre-TCR complex consisting of a productively rearranged β chain, an invariant α chain, known as preTα, and CD3 components. Only cells that have a functional pre-TCR survive the transition from DN3 to DN4, a process known as β-selection. Since successful rearrangement of the β chain results in an abrogation of rearrangement at the γ or δ loci the DN4 cells are destined to become α/β T cells (reviewed by Fehling, Gilfillan et al. 1999).

The nature of the blood-borne progenitors that colonise the thymus is not yet resolved. It is clear that thymic seeding progenitors constitute the DN1 population, but since DN1 cells are heterogeneous, both phenotypically and functionally, the true identity of the thymic settling cell is ambiguous. The ability of DN1 cells to generate α/β as well as γ/δ T-cells is well documented. Recent evidence suggests that T-cell activity resides exclusively in the $CD117^+$ fraction of the DN1, a phenotype that resembles an early haematopoietic progenitor (Matsuzaki, Gyotoku et al. 1993; Allman, Sambandam et al. 2003). These cells, termed the "early thymic progenitor" (ETP), are thought to be derived via a CLP independent pathway (Allman, Sambandam et al. 2003). Since bone marrow progenitors must seed the thymus via the blood, it is interesting to note that the CLP has not always been detected in the blood (Schwarz and Bhandoola 2004). A bone marrow analogue to the ETP has been described (Perry, Wang et al. 2004).

The double negative 1 $CD117^+$ population (ETP) is not a homogeneous progenitor pool, but instead contains a small fraction of cells that express CD135, and by this cell surface phenotype even more closely resemble the multipotent progenitor population of the bone marrow. This fraction of cells has been called the "thymic seeding progenitor" (TSP) and is believed to be the most immature cell type that colonises the thymus. Upon exposure to the thymic microenvironment, the TSPs generate the rest of the ETP pool and further progress along the T-cell developmental path (Sambandam, Maillard et al.

2005; Tan, Visan et al. 2005). Downregulation of CD135 is accompanied by the loss of B-cell potential (Sambandam, Maillard et al. 2005; Tan, Visan et al. 2005).

A knock-in of an enhanced green fluorescent protein (EGFP) into the CC chemokine receptor 9 (CCR9) gene identified yet another multipotent precursor in the $CD117^+$ fraction of DN1 cells (Benz, Bleul, 2005). Heterogeneity amongst the ETP population was illustrated by the wide-ranging expression levels of EGFP. The $EGFP^{high}$ fraction of the ETP generated T-, B-, NK- and myeloid cells *in vitro*, whereas the $EGFP^{low}$ population produced T-cells with quicker kinetics, but completely lost the ability to develop B-cells, whilst maintaining the NK and myeloid potentials. At the single cell level, the $EGFP^+$ cells generated T-, B-, and NK-cells *in vitro*. Since the ETP $EGFP^+$ population gave rise to mature cells of multiple haematopoietic lineages, it was termed the "thymic multipotent progenitor" (TMP). In a subsequent study, it was shown that the TMP exhibited cell surface expression of CD135, therefore overlapping with the population of cells described by Bhandoola's group (Heinzel, Benz et al. 2007).

However, a phenotypic dissection of the DN1 population according to the expression of heat stable antigen (HSA, CD24) and CD117 demonstrated existence of separate progenitor populations with distinct functional potentials (Porritt, Rumfelt et al. 2004). The T-cell activity was not only restricted to the $CD117^+$ fraction, the ability to generate T-cells was detected in the $CD117^-$ fraction of DN1 thymocytes, by a recently described *in vitro* culture system (Porritt, Rumfelt et al. 2004). Interestingly, the B-cell potential of the $CD117^+$ fraction was not revealed even under favourable culture conditions. Instead, the capacity to generate B-cells was limited to the DN1 $CD117^{low}CD24^+$ and the DN1 $CD117^-CD24^+$ populations. This discrepancy might have arisen due to the cell isolation procedure employed by these authors, where an initial density gradient was used, thus introducing an additional variable into the study.

These data suggest that the murine thymus is seeded by a multipotent progenitor population, which commits to the T-cell lineage upon exposure to the thymic microenvironment. In addition, the thymus can also be seeded by alternative progenitor pools, which can converge to generate T-cells, or diverge to yield a different outcome.

Factors Regulating T-cell Development

Migration

A pre-requisite for successful T-cell development is the thymic colonisation by progenitor cells. Essential steps in the voyage of the cell, such as adhesion to endothelia and transmigration, are aided by the expression of proteins called integrins (Hynes 2002). All integrins are obligatory αβ heterodimers. The α subunits vary in size between 120 and 180 kDa and are each non-covalently associated with a β subunit, varying from 90 to 110 kDa. Integrins are the major metazoan receptors for cell adhesion to extracellular matrix proteins, such as fibronectin and laminin and in vertebrates play a major role in certain cell-cell associations. In addition to mediating cell adhesion, integrins link transmembrane connections to the cytoskeleton and activate many intracellular signalling pathways resulting in cytoskeletal reorganisation and changes of gene expression affecting proliferation, differentiation and survival of cells. Figure 1.4 shows the complete mammalian integrin subunit set, comprising of 8 β and 18 α subunits, so far known to assemble into 24 distinct integrins.

Mutations in integrin receptors have alluded to their involvement in haematopoiesis. Adult mice, deficient in β1 integrin, showed impairment of HSC homing to primary haematopoietic tissues. Reconstitution analysis of lethally irradiated mice revealed that β1-null HSC were not radioprotective due to an obvious defect in bone marrow

colonisation. A complete loss of α5 and reduced expression of α4 and α6 integrin subunits accompanied the deletion of β1 integrin subunit (Potocnik, Brakebusch et al. 2002). Cell surface expression of various integrin subunits, such as α4 integrin, α5 integrin, α6 integrin and β1 integrin, has been described on developing thymocytes along with the expression of their respective ligands, fibronectin and laminin on the thymic epithelia (Prockop, Palencia et al. 2002), suggestive of integrin involvement in progenitor thymocyte migration during differentiation. The involvement of α4 integrin in adult T-cell development was further revealed by blastocyst complementation experiments, where α4 integrin null progenitor cells failed to give rise to mature T-cells due to the inability of thymic colonisation (Arroyo, Yang et al. 1996).

Figure 1.4 The Integrin Receptor Family.

Integrins are αβ heterodimers: each subunit crosses the membrane once, with most of each polypeptide (>1600 amino acids in total) in the extracellular space and two short cytoplasmic domains (20–50 amino acids). The figure depicts the mammalian αβ subunits and their associations; 8 β subunits can assort with 18 α subunits to form 24 distinct integrins. These can be considered in several subfamilies based on evolutionary relationships (colouring α of subunits), ligand specificity and, in the case of β2 and β7 integrins, restricted expression on white blood cells. α subunits with grey hatching or stippling have inserted I/A domains. Such α subunits are restricted to chordates, as are α4 and α9 (green) and subunits β2- β8. In contrast, α subunits with specificity for laminins (purple) or RGD (blue) are found throughout the metazoa (Taken from Hynes R.O. 2002).

Intracellular signalling events can modulate the affinity or avidity of integrins, which in turn are crucial for cell translocation. This in part can be mediated by signals derived via chemokine receptors. Expression of C-C chemokine receptors-7 and -9 (CCR-7 and CCR-9), as well as C-X-C chemokine receptor 4 (CXCR-4) on various subsets of progenitor thymocytes alludes to the involvement of these receptors in early thymocyte migration and differentiation (Misslitz, Pabst et al. 2004). Thymocyte specific deletion of CXCR-4 resulted in an early developmental arrest (Plotkin, Prockop et al. 2003). The inability of cells lacking CXCR-4 to differentiate past the DN1 stage illustrates that migration into the cortex and interaction with signals found in the cortical microenvironment are essential processes for continuous T-lymphocyte generation. Deficiency of CCR-7 was mirrored in altered thymic architecture, reduced thymic cellularity and impaired thymocyte development (Misslitz, Pabst et al. 2004), which illustrates a tight connection between thymocytes and the organ where they develop.

Transmembrane Signalling and Growth Factors

A number of complex cellular and molecular interactions will ultimately determine the fate of all cells. Signalling through the Notch receptor has been shown to influence cell fate decisions in many different organisms (reviewed in Artavanis-Tsakonas, Rand et al. 1999). Of the four mammalian Notch proteins, Notch 1, 2 and 3 are expressed in the thymus along with ligands Jagged 1 and 2 and Delta like-1 and -4 (Radtke, Wilson et al. 2004). Upon ligand engagement, proteolytic cleavage events free the intracellular domain of Notch (NotchIC, ICN), which translocates to the nucleus where it binds the helix-loop-helix transcription factor CSL/RBP-J. In the absence of ICN, CSL/RBP-J forms a repressor complex thus inhibiting transcription, whereas upon interaction with

ICN, corepressors are displaced and coactivators are recruited, leading to transcriptional activation. Targets of Notch signalling include Hairy/Enhancer of Split (HES), Deltex (Dtx) and more specifically for the T-lineage – preTα (Reizis, Leder, 2002).

The Notch signalling pathway seems to be crucial for a number of developmental events during T-cell development. The most notable is the initial T-cell lineage decision. By overexpression of NotchIC in haematopoietic progenitors, T-cell development can be initiated in the bone marrow at the expense of B-cell development, without any obvious defect in myelopoiesis (Pui, Allman et al. 1999). Induced inactivation of Notch-1 results in a developmental block at the DN1 stage of thymic development, generating thymic B-cells phenotypically resembling immature B-cells normally found in the bone marrow (Radtke, Wilson et al. 1999). Moreover, Notch-1 deficient precursor cells adopt a B-cell fate in the thymus (Wilson, MacDonald et al. 2001). More recently, an absolute dependence on Notch signalling in generating the ETP subset was demonstrated (Sambandam, Maillard et al. 2005; Tan, Visan et al. 2005).

An indirect approach also illustrated critical dependence of early thymic progenitors on Notch-1 receptor signalling (Koch, Lacombe et al. 2001; Visan Tan et al. 2006). This was achieved by ectopically expressing Lunatic Fringe under the control of the Lck proximal promoter. Lunatic Fringe increases the affinity of the Notch-1 receptors for the Delta like ligands, thereby generating cells, which have an increased capacity to bind Delta like ligands. In this system, T-cell development was impaired, but not completely blocked, instead, increased B-cell development was observed in the thymus. This can be interpreted as the inability of the earliest thymic seeding progenitors to locate to microenvironments supplying the Notch-1 signals, because, these sites are occupied by competitor cells, which have a higher affinity for Delta like ligands. Thus, seeding progenitors are excluded from the correct developmental niche and generate B-cells due

to a lack of Notch-1 signalling, therefore illustrating the importance of Notch-1 signalling during T-cell development, and additionally demonstrating a role for Notch-1 signalling in T- versus B-cell lineage specification (Koch, Lacombe et al. 2001; Visan Tan et al. 2006).

Notch-1 signals have also been suggested to promote the α/β over the γ/δ T lineage and to influence the CD4 vs CD8 T lineage decision (Robey, Chang et al. 1996; Washburn, Schweighoffer et al. 1997). However, the role of Notch-1 signalling remains controversial beyond T-cell commitment given that Notch-1 deletion after the checkpoint of β-selection failed to influence subsequent stages of thymocyte development, whereas inactivation of Notch-1 at DN2/3 stage affected α/β but not γ/δ T-cell development due to impairment in Vβ to DJβ rearrangements (Wolfer, Bakker et al. 2001; Wolfer, Wilson et al. 2002).

Signalling through Notch alone, however, is not sufficient to drive T-cell development. The critical role of interleukin-7 (IL-7) is revealed by a dramatic loss in numbers of thymocytes and mature T-cells in mice lacking IL-7 (von Freeden-Jeffry, Vieira et al. 1995) or the respective receptor (IL-7Rα, CD127) (Peschon, Morrissey et al. 1994). In mice carrying homozygous mutations in this cytokine or cytokine receptor gene there is a partial block at the DN2 stage of thymic development. The requirement of this cytokine seems to be an early event, since DN1 cells cannot progress to the next stage of maturation without IL-7 in an *in vitro* culture system (Balciunaite, Ceredig et al. 2005).

Signalling through the c-kit receptor tyrosine kinase (c-kit, CD117) has been shown to promote differentiation of progenitor thymocytes (Moore and Zlotnik 1997; Balciunaite, Massa et al. 2006). Mutations in the *c-kit* gene or the gene encoding its ligand, *stem cell factor (SCF)* yield reduced numbers of thymocytes, even though T-cell development is

not completely abrogated (Rodewald, Kretzschmar et al. 1995). Moreover, blocking antibodies against c-kit do not extinguish the ability of thymically derived progenitors to generate T-cells (Matsuzaki, Gyotoku et al. 1993). This would suggest that an alternative mechanism compensates for the lack of c-kit signalling in the thymus or that an unconventional progenitor population exists which has no reliance on the c-kit–SCF signalling pathway.

A candidate signalling mechanism, which could compensate for the absence of functional c-kit signalling, is through the flk-2 receptor tyrosine kinase (flk-2, flt-3, CD135), which promotes differentiation of progenitor thymocytes (Moore and Zlotnik 1997; Maillard, Sambandam et al. 2005). Mice carrying a homozygous mutation in the *flk-2* gene show severe impairment in B-cell development but the numbers and relative frequencies of thymocyte subpopulations are normal (Mackarehtschian, Hardin et al. 1995). On the other hand, there was a marked decrease in the competitive repopulating ability of *flk-2*$^{-/-}$ haematopoietic precursors to generate T-cells. The dual role of c-kit and flk-2 signalling was demonstrated in the *c-kit*$^{-/-}$*flk-2*$^{-/-}$ double mutant where a dramatic reduction in T-cell numbers was evident (Mackarehtschian, Hardin et al. 1995). These data suggest that signal transduction through both receptors is necessary for T-cell development and does not preclude the possibility that signalling through either or both receptors is required in generating T-lineage precursors at a pre-thymic stage.

Additionally, c-kit derived signals converge with signals generated by receptors containing the common cytokine receptor γ chain (CD132), since a combinatorial mutation of the *c-kit* and the γ_c genes revealed a complete absence of thymocytes (Rodewald, Ogawa et al. 1997). This suggests that distinct signalling pathways must combine to facilitate the proper development of T-cells. The critical role of the pre-TCR

signalling is best exemplified through targeted disruption of RAG-1/2 or preTα, all of which result in a severe arrest in α/β T-cell development at the DN3 stage (Mombaerts, Iacomini et al. 1992; Shinkai, Rathbun et al. 1992; Fehling, Krotkova et al. 1995).

Two evolutionary conserved signalling cascades, the Wingless/β–catenin pathway and the Hedgehog pathway, play pivotal roles in a number of developmental systems and have been shown to participate in the development of T-lymphocytes (as reviewed by Varas, Hager-Theodorides et al. 2003; Staal and Clevers 2005). The secreted Wingless (Wnt) proteins bind the Frizzled (Fz) receptors and the low density lipoprotein receptor-related proteins-5 and -6 (LRP-5 and LRP-6), forming a unit that initiates the Wnt signalling pathway. This signalling cascade leads to the stabilisation of β–catenin, which translocates to the nucleus where it engages with the lymphoid enhancer factor-1 (LEF-1) and T-cell factors-1, -3 and -4 (TCF-1, TCF-3 and TCF-4) and promotes the expression of T-lymphoid specific genes, such as CD3ε, TCRα, TCRβ, TCRδ and CD4. In the absence of Wnt signalling, β–catenin is phosphorylated and targeted for proteosomal degradation. The relevance of this signalling cascade in T-cell development is exemplified by a targeted mutation of the *TCF-1* gene, which severely affects stages of immature thymocyte differentiation where proliferation occurs (Schilham, Wilson et al. 1998), however does not completely abrogate T-cell development. The partial development of T-cells can be explained by a functional redundancy between TCF-1 and LEF-1, however once LEF-1 is removed along with TCF-1, a complete arrest of T-cell differentiation is observed (Okamura, Sigvardsson et al. 1998).

The family of Hh proteins consists of three secreted proteins, Sonic (Shh), Indian (Ihh) and Desert (Dhh), which bind the Patched (Ptch) receptor that in the absence of Hh acts as a negative regulator of signalling by inhibiting a downstream signal transducer

smoothened (Smo). Hh proteins bind to and inhibit Ptch, thereby allowing signal transduction via Smo, which ultimately causes nuclear localisation and activation of the Gli family of transcription factors influencing cell survival and proliferation. Deletion of the smoothened receptor during early stages of T-cell development revealed a dramatic decrease in thymocyte numbers and a near absence of the ETP population (El Andaloussi, Graves et al. 2006). This suggests that T-cell development can proceed, albeit heavily impaired, via an ETP independent pathway.

Gene Regulatory Networks

Thymocyte development is critically dependent on extracellular signals provided by the stromal compartments of the thymus. Ultimately, however, cellular differentiation is defined by changes in the expression pattern of required genes. This is controlled by nuclear transcription factors, which specifically bind gene regulatory elements to induce or repress gene expression. Transcription factors present within a cell act in regulatory networks to establish various patterns of gene expression, changes in which will eventually define cellular differentiation and ultimately the functional capacity of a cell. A number of lineage specific transcription factors have been identified in the haematopoietic system, these include paired box gene 5 (Pax-5), inhibitor of DNA binding 2 (Id-2) and GATA binding protein 1 (GATA-1), which are essential for the development of B-, natural killer and erythroid lineages, respectively (Nutt, Heavey et al. 1999; Mansouri, Mori et al. 1999; Pevny, Simon et al. 1991). For the T-cell lineage, such master regulator has so far been intangible. Instead, it is believed that specification and commitment to the T-cell lineage depends on an intricate balance of transcription factors, most of which are shared between at least one other haematopoietic

differentiation program. Therefore, the critical feature of the T-cell developmental program is the precise timing and amount of transcriptional regulator expression.

One factor, GATA binding protein 3 (GATA-3), has been shown to be essential and specific to the T-cells lineage and regulates the development of early thymocytes. Blastocyst complementation experiments revealed that GATA-$3^{-/-}$ embryonic stem (ES) cells failed to contribute to the double negative thymocyte population (Ting, Olson et al. 1996), which may suggest that GATA-3 elicits its function pre-thymically and that specification to the T-lineage can occur without the instructive signals from the thymic microenvironment. Interestingly, overexpression of GATA-3 in haematopoietic progenitors did not result in the enhancement of T-lineage differentiation, conversely, an arrest at the earliest stage of T-cell development was seen in foetal thymic organ cultures (Anderson, Hernandez-Hoyos et al. 2002), suggesting that GATA-3 cannot be classified as a definite switch promoting T-cell development, and more importantly, that the correct concentration of this transcription factor is crucial for adequate T-cell development. The exclusivity of GATA-3 in the T-cell lineage has recently been questioned by the finding that natural killer (NK) cells developing in the thymus are also dependent on GATA-3 (Vosshenrich, Garcia-Ojeda et al. 2006). This experiment was inconclusive however. The irradiation chimeras reconstituted with GATA-3 deficient haematopoietic precursors failed to generate thymically derived NK-cells. This can be interpreted as the inability of GATA-3 haematopoietic precursors to colonise the thymus, which would be in line with the results of Ting *et al.*, instead of a direct role of GATA-3 in driving the thymic NK-lineage differentiation.

T-cell specific factor 1 (TCF-1) is expressed exclusively in the T-lineage of the haematopoietic system, and is required for the proper development of this lineage, however, a functional redundancy with lymphoid enhancer factor 1 (LEF-1), illustrates

only a partial dependence of progenitor thymocytes on this molecule. Consequently it seems that establishment of the T-cell identity is a result of a multitude of inputs at various stages of development, some acting in a positive fashion to promote the T-lineage, others acting as negative regulators of alternative haematopoietic lineages, thus indirectly favouring T-cell development.

The importance of the ICN/CSL/RBP-J transcriptional complex in T-cell development was discussed earlier. The exact mechanism by which this complex elicits its function has not been shown directly, but it seems to act in dual fashion. Firstly, by possible inhibition of *Pax-5* transcription and therefore inhibition of B-cell development, and secondly, by initiation of T-cell specific genes, such as *preTα* (Reizis, Leder, 2002), therefore suggesting that commitment to the T-lineage is paralleled to the shedding of alternative haematopoietic lineages. It has also been proposed that CD117 (c-kit) expression is induced after the exposure to an environment rich in Notch ligands (Massa, Balciunaite et al. 2006). Since the earliest thymic progenitor cells with the most potent T-cell developmental potential all express CD117, it would be predicted that these cells have already received a Notch signal prior to thymic entry, or the earliest thymic immigrants are negative for CD117 expression upon entrance to the thymus, and upregulate this receptor following the exposure to the thymic microenvironment rich in Notch ligands. An additional explanation could be the possibility that CD117 expression can be regulated by another signalling event.

Member of the ETS family of transcription factors, PU.1, effects the development of the B- and myeloid lineages in a graded fashion (DeKoter and Singh 2000), where low levels of PU.1 expression favours B-cell development and higher levels induce the development of macrophages. In the T-cell lineage, disruption of PU.1 led to a severely reduced number of mature T-cells, with a delay in developmental kinetics (McKercher,

Torbett et al. 1996). An additional study illustrated that colonisation of the thymus by PU.1 deficient progenitors was not affected, however subsequent development along the T-lineage was blocked (Spain, Guerriero et al. 1999), suggesting a role of PU.1 at the earliest stage of thymocyte differentiation, to induce the expression of interleukin 7 receptor α chain (CD127), for example, as is the case during B-cell development (Singh, Medina et al. 2005). However, enforced expression of PU.1 resulted in a developmental block at the DN3 stage of progenitor development (Anderson, Weiss et al. 2002), thus demonstrating that precise timing of expression of a transcriptional regulator is a prerequisite for successful developmental progression. Inhibition of T-cell development could be due to the negative effect of PU.1 on *Rag-1* and *Rag-2* gene transcription (Anderson, Hernandez-Hoyos et al. 2002).

The reliance on the products of the *E2A* gene, E12 and E47, is shared between the developmental programs of T- and B-cells. The absolute requirement of E2A in B-cell development is revealed by the complete absence of mature B-cells in mice lacking the *E2A* gene (Bain, Maandag et al. 1994). In T-cells, lack of E2A products leads to a partial block in T-cell development at the earliest stage of thymocyte differentiation (Bain, Engel et al. 1997). E12 and E47 can act on the target sites either as homodimers or as heterodimers and in addition with another member of the basic helix-loop-helix (bHLH) protein family HEB. Deficiency of HEB also leads to a partial block in thymocyte differentiation (Barndt, Dai et al. 1999), but can be compensated by the actions of E2A proteins, therefore illustrating redundancy between these closely related molecules. However, a dominant negative form of HEB, which forms non-functional heterodimers with the E2A proteins, blocks development of T-cells at the DN3 stage of differentiation (Barndt, Dai et al. 2000). The action of these bHLH transcription factors can be antagonised by the inhibitors of DNA binding (Id) proteins, which in turn are

required for the development of alternative haematopoietic lineages, therefore mistimed expression of Id proteins can lead to an abort of the T-cell lineage and acquisition of a different fate (Heemskerk Blom et al. 1997). This illustrates that correct combination of transcriptional regulators is crucial for the establishment of the T-cell lineage.

These examples do not fully reflect the complexity of genetic inputs required for the establishment of T-cell identity. T-cell lineage specification also depends on members of the Runx family of transcriptional regulators, members of the Ikaros family of transcription factors, members of the T-box factor family and members of the HOX gene family (as reviewed in Rothenberg and Taghon 2005). These few examples aim to illustrate the general principles of gene regulatory networks required for correct T-cell development. The correct timing of transcriptional regulator, as well as the amount of a given factor is of uttermost importance in guiding the T-cell developmental process. Correct combination of transcription factor complexes yields the desired outcome, at the expense of an alternative haematopoietic lineage.

Transcriptional Signatures

Ultimately, the pattern of gene expression in a given cell will determine its functional attributes. This suggests that gene expression signatures can predict the identity of a given cell along with its functional capabilities. A genome-wide screen of human foetal HSCs, murine foetal HSCs, murine adult HSCs, murine adult neural stem cells (NSC) and murine embryonic stem cells (ESC) revealed several key features of stem cell biology (Ivanova, Dimos et al. 2002). Comparing human and murine foetal liver HSC transcriptomes revealed that 39% of genes expressed in human HSCs were co-expressed in the murine equivalent, which identifies evolutionary conserved elements in the HSC genetic program between human and mouse. Comparing adult and foetal HSCs revealed

that more than 70% of all HSC related gene products were co-expressed, therefore identifying a set of genes defining a HSC at the molecular level. Interestingly, there was not a complete overlap of genes expressed between foetal and adult HSCs, which may suggest that foetal and adult haematopoiesis differ. Comparison of gene expression patterns between the two adult stem cell populations and the embryonic stem cell population recognised a set of commonly expressed elements, which can be classified as the functional hallmark of all stem cells, namely, the ability to balance self-renewal and differentiation. Importantly, each stem cell population expressed a set of non-overlapping, distinct genes, on the basis of which a specific stem cell population could be defined (Ivanova, Dimos et al. 2002). A similar study extracted 216 genes, which were co-expressed between adult HSCs, adult NSCs and ESCs (Ramalho-Santos, Yoon et al. 2002). These authors provided a similar interpretation, the commonly expressed genes constituted the core stem cell functional properties, or "stemness", that underlie self-renewal and the ability to generate differentiated progeny (Ramalho-Santos, Yoon et al. 2002). These experiments demonstrate that functional properties of a given cellular population can be reflected in the transcriptional signature of that population and in addition specific sets of genetic clusters can be used to define a cellular population at the molecular level.

Aim of Study

At this moment it is still unclear how T-cell lineage specification is initiated, sustained and accomplished in the adult mouse. It is unclear which exact progenitor cells colonise the thymus and thus give rise to T-cells, and ultimately what are the origin of these cells in the bone marrow. The exact intermediate stages in the bone marrow are still unresolved. It has not been shown directly that the common lymphoid progenitor constitutes a direct precursor to the DN1 progenitor thymocyte population. Currently the physiological importance of the common lymphoid progenitor in the generation of the T-lineage is under question.

In this study, an analysis of the earliest stages of T-cell development will try and provide some clues to the nature of the T-cell developmental process. Initially, a careful phenotypic dissection of double negative thymocytes will aim to reveal the composition of various developmental populations. Based on these phenotypic definitions, would new populations be identified, and would it be possible to align them with already well-defined stages of T-cell development? To further understand the process of T-cell development, purified progenitor populations will be isolated and characterised by a genome wide screen. Would a genetic signature of each population reflect the functional attributes of these cells? Would it be possible to relate each progenitor population based on their molecular fingerprints? In addition the gene expression study will try to aid in understanding the relationship between already characterised bone marrow progenitor cells and the intermediate stages in T-cell development in the thymus. An additional tool, in the form of a fluorescent reporter system will aim to facilitate in understanding the relationship between thymic progenitor cells and intermediate stages of development in the bone marrow. Additionally, this reporter system will aim to reveal the point of commitment to the T-cell lineage.

Chapter 2

Materials and Methods

Mice

C57BL/6, C57BL/6 CD45.1, BALB/c, CBA and C3H/He wild-type mice were bred and housed under specific pathogen free conditions at the MRC National Institute for Medical Research (NIMR). *Recombinase activating gene 2* deficient (Rag2-/-) mice, on the C57BL/6 background, were generated by Shinkai et al., (Shinkai, Rathbun et al. 1992). *cd127* deficient (CD127-/-) mice, on the C57BL/6 background, were generated by von Freeden-Jeffry et al., (von Freeden-Jeffrey, Vieira et al. 1995). The R26REYFP strain was made by Srinivas et al., (Srinivas, Watanabe et al. 2001), who inserted the enhanced yellow fluorescent protein (EYFP) cDNA, preceded by a loxP-flanked stop sequence, in to the ROSA26 locus. This strain was then crossed to protamine-1::Cre strain (O'Gorman, Dagenais et al. 1997) to generate mice constitutively expressing EYFP (B6.EYFP), or to hCD2::iCre strain (de Boer, Williams et al. 2003) to generate the reporter strain (hCD2::iCre EYFP reporter).

Lck deficient/*Fyn* deficient (Lck-/-Fyn-/-) mice were a kind gift from R. Zamoyska (NIMR). *FoxN1* deficient (nude) and *CD90.1* mice on the BALB/c background were a kind gift from J. Langhorne (NIMR). *cd132* deficient/*recombinase activating gene 2* deficient (CD132-/-Rag-2-/-) mice were a kind gift from G. Stockinger (NIMR). Where possible, mice were female and between 4-6 weeks of age at the start of experiments.

Cell and Tissue Preparation

To achieve single cell suspensions of thymocytes and splenocytes, thymi and spleens were isolated and mechanically disrupted. Bone marrow was isolated by flushing the femoral long bone with ice cold phosphate buffered saline (PBS) and gentle passage through a 70 micron nylon mesh (BD Falcon). Blood was isolated by cardiac puncture of lethally anesthetised mice (40mg Pentobarbitone – Animalcare) with a heparinised

syringe (Heparin - Sigma-Aldrich). To remove erythrocytes, bone marrow, splenocyte and blood suspensions were centrifuged through a 1.074 g/ml Percoll (Amersham Biosciences) solution. Cell numbers were determined by counting viable cells using Trypan-blue exclusion in an improved Neubauer chamber.

Lineage depletion was achieved by incubating single cell suspensions with anti-Fc-receptor II/III monoclonal antibody (mab) CD16/CD32 (hybridoma supernatant, clone 2.4G2) to block any non-antigen specific binding of antibodies and subsequent incubation with subsaturating concentrations of biotinylated anti-CD8α (53-6.7), anti-CD3ε (500A2), anti-CD19 (1D3), anti-NK1.1 (PK136), anti-CD11b (M1/70) and anti-TER119 (Ly76) (all eBioscience), followed by a second labelling step with paramagnetic streptavidin conjugated microbeads (Miltenyi Biotech). Microbead labelled cells were finally removed by passing the cell suspension through a magnetic field. The resulting eluant contained lineage negative cells. In some cases, during lineage depletion of bone marrow and blood, anti-CD19 antibody was replaced by anti-CD45R (B220) antibody (RA3-6B2) (eBioscience) and anti-Ly6G (Ly-6G) antibody (RB6-8C5) (eBioscience) added into the lineage depletion cocktail. To remove natural killer cells from thymocyte preparations of CBA, BALB/c and C3H/He mouse strains, anti-NK1.1 antibody was replaced by anti-CD49b (DX5) antibody (eBioscience).

Flow Cytometry

Cell concentrations were adjusted to 3 x 10^7 cells/ml for preparative flow cytometry or 4 x 10^6 cells/ml for analysis. Stainings were performed using Pacific Blue conjugated anti-CD45R (B220) (RA3-6B2) and anti Ly6A/E (Sca-1) (D7) (bought from BioLegend). Fluorescein isothiocyanate (FITC)-conjugated antibodies used were anti-CD8α (53-6.7), anti-CD3ε (500A2), anti-CD90.2 (Thy1.2) (30-H12), anti-CD24

(heat stable antigen, HSA) (M1/69), anti-CD45R (B220) (RA3-6B2), anti-CD4 (GK1.5) and anti-CD44 (IM7) (all Pharmingen, apart from CD8α, bought from eBioscience). Phycoerythrin (PE)-conjugated antibodies used were anti-CD4 (GK1.5), anti-CD127 (IL-7Rα) (A7R34), anti-Ly6G (RB-8C5), anti-CD11b (Mac-1) (M1/70), anti-NK1.1 (PK136), anti-TER119 (Ly76), anti-CD19 (1D3), anti-CD25 (3C7), anti-CD117 (c-kit) (ack45), anti-CD45.1 (A20) and anti-CD45R (B220) (RA3-6B2) (all Pharmingen, apart from CD127 and NK1.1, bought from eBioscience). Peridinine chlorophyll protein (PerCP)-Cy5.5 conjugated antibody used was anti-CD25 (PC61) (Pharmingen). Allophycocyanin (APC)-conjugated antibodies used were anti-CD90.2 (53-21), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD117 (c-kit) (2B8), anti-T-cell receptor (TCR) beta chain (H57-597) and anti-CD45R (B220) (RA3-6B2) (all eBioscience, apart from CD90.2 and CD25, bought from Pharmingen). APC-Cy7-conjugated antibody used was anti-CD44 (IM7) (eBioscience). Biotin-conjugated anti-CD117 (c-kit) (2B8) (Pharmingen) was revealed with PE-streptavidin (Southern Biotechnology Associates) or APC-streptavidin (Molecular Probes). Biotin-conjugated anti-CD8α (53-6.7) (eBioscience) was revealed with PerCP-streptavidin (Pharmingen). Biotin-conjugated anti-CD25 (7D4) (Pharmingen) was revealed with AlexaFluor 610-streptavidin (Molecular Probes). Samples were analysed on an analytical flow cytometer (Calibur (Becton Dickinson Europe) or Cyan (Dako Cytomation) or LSR (Becton Dickinson Europe)) and results were analysed using FlowJo software (Tree Star, Inc). Preparative flow cytometry was performed on a dual-laser high-speed fluorescence activated cell sorter (FACS) (MoFlo flow cytometer, Cytomation). For RNA preparation, equal numbers of cells were sorted directly into 600 µl TRI Reagent®.

RNA Extraction and Complementary DNA Preparation

RNA was extracted by following the protocol of Chomczynski and Sacchi (Chomczynski and Sacchi 1987). Briefly, cells were homogenized in TRI Reagent® (Molecular Research Center, Inc) supplemented with 1/10 v/v polyacryl carrier (Helena Biosciences), phases were separated by supplementation of bromochloropropane (BCP, Molecular Research Center, Inc) and brief centrifugation. RNA was precipitated from the aqueous phase with isopropanol and washed twice with ethanol. Finally, RNA was air-dried and solubilised at 1 ng/µl. The quality of RNA was assessed on a BioAnalyzer 2100 (Agilent). cDNA was prepared by reverse transcription of the RNA sample using SuperScript II® reverse transcriptase (Invitrogen) according to the instructions of the manufacturer.

Polymerase Chain Reaction and Agarose Gel Electrophoresis

Polymerase chain reactions (PCRs) were carried out in a final reaction volume of 20 µl containing 1x reaction buffer, 15 mM magnesium chloride (both Abgene), 200 µM dNTP (Amersham Biosciences), 0.5 µM 5' and 3' primer, 0.5 ng template cDNA and 0.2 U ThermoPrime DNA polymerase (Abgene). All reactions were carried out using PC-200 thermal cycler (MJ Research) for 35 cycles. All amplifications were in the linear phase. Primer sequences and PCR parameters are given in Table 2.1. After PCR, all products were analysed by analytical agarose gel electrophoresis in 1.5% agarose gels visualised by ethidium bromide staining. After separation the gels were photographed on a UVP BioDoc-It system.

Quantitative Real Time Polymerase Chain Reaction

Reactions were performed by mixing 2 x QuantiTect Probe PCR Master Mix (Qiagen) 20 x Primer Mix (Qiagen), 20 x Quanti Probe (Qiagen), and cDNA template equivalent

to 1 x 10^3 cells to a final volume of 20µl. All reactions were done in triplicates for 40 cycles on an ABI 7900 Sequence Analyzer (Applied Biosystems) according to manufacturer's instructions. Data was analysed by the Standard Curve Method (Applied Biosystems), and differences in cDNA input were compensated by normalising against *acidic ribosomal phosphoprotein* (Arbp) expression levels. Primer/probe sequences are given in Table 2.2.

Microarray Analysis

Complementary RNA Synthesis and Hybridisation

RNA from eight independent sorting experiments was pooled to generate the starting material. The starting RNA amount did not exceed 1µg therefore a two-cycle amplification procedure was used, as recommended by the manufacturer (Affymetrix). All procedures were conducted by the Microarray Core Facility at the MRC National Institute for Medical Research. Briefly, total RNA was spiked with Poly-A RNA Spike (Affymetrix) consisting of *lys, phe, thr,* and *dap* genes from *B. subtilis*, to later control for amplification efficiency, and reverse transcribed using SuperScript II and a T7-Oligo(dT) Promoter Primer in the first-strand cDNA synthesis reaction (Two-Cycle cDNA Synthesis Kit, Affymetrix). Following RNase H – DNA polymerase I-mediated second-strand cDNA synthesis, the double stranded cDNA served as a template for the first cycle of *in vitro* transcription (IVT) (MEGAscript® T7 Kit, Ambion, Inc.). The resulting unlabelled cRNA was then reverse transcribed in the first-strand cDNA synthesis step of the second cycle using SuperScript II and random primers (Two-Cycle cDNA Synthesis Kit, Affymetrix). Subsequently, the T7-Oligo(dT) Promoter Primer was used to generate double stranded cDNA template containing T7 promoter sequences. The resulting double stranded cDNA was amplified and labelled using biotinylated nucleotides in the second IVT reaction (IVT Labelling Kit, Affymetrix), to

yield labelled cRNA. All reactions were done in a GeneAmp 9700 PCR System (Applied Biosystems). The biotinylated cRNA was cleaned by the Sample Cleanup Modules (Affymetrix), fragmented by metal-induced hydrolysis and hybridised to the Mouse 430A_ 2.0 GeneChip (Affymetrix). The control oligonucleotide B2 was added to each chip hybridisation reaction, according to the guidelines of the manufacturer (Affymetrix). The B2 oligonucleotide serves as a positive hybridisation control and is used by the GeneChip Operating Software (GCOS) to place a grid over the image in order to define the probe area. In addition, *bioB*, *bioC*, *bioD*, and *Cre* were added at specific concentrations to control for hybridisation efficiency. Staining with PE-streptavidin and washing was performed using an automated fluidics workstation (Affymetrix), and the arrays were immediately scanned on an Affymetrix GeneChip Scanner, generating an image of the expression data.

Identification of Expressed Genes

The methods for analysis of the chip image were those recommended by the manufacturer (Affymetrix), which implemented the MAS 5.0 transformation algorithm. Two primary parameters are of interest for each gene on the chip, namely, the detection call and the signal intensity. The Detection algorithm of GCOS (Affymetrix) generated a Detection ρ-value, which provided boundaries for defining Present, Marginal or Absent calls. To determine the Detection p-value, first, a Discrimination score (R) for each probe pair was calculated as follows:

$R = (PM - MM) / (PM + MM)$

where PM = Perfect Match and MM = Mismatch.

The Discrimination score (R) was next compared to the default threshold ($\tau = 0.015$) and the One-Sided Wilcoxon's Signed Rank test employed to generate the Detection

p-value by assigning each probe pair a rank based on how far the probe pair Discrimination score was from τ. If the PM is much larger than the MM, the Discrimination score for that probe pair will be close to 1.0. If the Discrimination scores are close to 1.0 for the majority of the probe pairs, the calculated Detection *p*-value will be lower (more significant). A lower *p*-value is a reliable indicator that the result is valid. Conversely, if the MM is larger than or equal to the PM, then the Discrimination score for that probe pair will be negative or zero. If the Discrimination scores are low for the majority of the probe pairs, the calculated Detection *p*-value will be higher (less significant). The Detection *p*-values below 0.05 were assigned a Present call, whereas values above 0.065 were assigned an Absent call.

The Signal intensity value was determined by taking the $\log_2$ of the difference in intensity between each matched/mismatched probe pair and applying the One-Step Tukey's Biweight Estimate to calculate the mean of the weighted intensity value for a probe set. The mean value was corrected back to linear scale and output as Signal.

Quality Control of Microarray Data

The *affyQCReport* R package run in BioConductor was implemented to assess the data quality of processed arrays. The non-normalised image data contained in .cell file of each array was assessed for PM intensities, 3':5' ratio of spiked-in and control genes, fluorescence of positive and negative border elements on the outer edges of the array, the "Centre of Intensity" (COI) position and the Spearman rank correlation coefficients. To determine positive and negative border elements, intensities for all border elements were collected for each array. Elements with intensity greater than 1.2 times the mean for that group were assumed to be positive controls. Elements with signal less that 0.8 of the mean were assumed to be negative controls. Elements falling between these cut

offs were not used for further calculations. The means and standard deviations of the intensities for each array should be comparable. Large variations in the positive control can indicate non-uniform hybridization or gridding problems. Variations in the negative controls indicate background fluorescence. To calculate COI, the mean fluorescent values for the left, right, top and bottom elements were calculated for positive and negative controls. The values were separated into positive and negative elements, and further divided by the 4 locations, and the COI for the controls calculated. Uniform hybridization is reflected in the COI for the positive elements located in the physical centre of the array, point 0.0,0.0. The COI for the negative elements reflects the uniformity of background fluorescence on the array, point 0.0,0.0 indicative of uniform background signal.

Data Processing and Analysis

The resulting information generated by the GCOS (Affymetrix) was output as a .txt file and applied to the GeneSpring Microarray Analysis Software (Silicon Genetics/Agilent) for processing and analysis. Individual arrays were initially normalised by dividing the fluorescence of each probe by the median fluorescence of a given array. Subsequently, fluorescence of each gene was normalised to the median fluorescence of that specific gene across all 3 microarrays. The resulting normalised data was first filtered on the Absolute detection call, genes registering a "Present" call were taken for further analysis. Next, gene expression values in the DN1 CD117 population were considered as baseline and compared with values obtained from the DN2 and DN3 populations. Genes exhibiting a 1.8 fold change in at least one instance were classified as regulated and were taken for further analysis. The Pearson correlation was used to group genes based on their expression levels, genes exhibiting similar expression levels were group

together. K-means clustering was used to group genes based on their expression patterns, genes exhibiting similar patterns of expression were assigned the same cluster. The Gene Ontology (GO) Consortium designations were used to identify genes involved in the same biological process.

Assessment of Developmental Potential

In vitro Co-cultures

From total double negative (DN) thymocytes, DN1 CD117 cells were sorted as CD44+CD25-CD117+CD45R- and DN1 CD45R cells were sorted as CD44+CD25-CD117-CD45R+. Progenitor cells were seeded at 1×10^3 cells/well into 6-well tissue culture plates containing a confluent monolayer of 3000 rad γ-irradiated cloned OP9-GFP cells or OP9-DLL1-GFP cells. At indicated time points, co-cultures were harvested by gentle pipetting and donor derived progeny analysed by flow cytometry, gating on GFP negative cells. For limiting dilution analysis, 96 replicate cultures containing 1, 2, 5, 10 and 20 cells were plated on OP9-GFP bone marrow stromal cell monolayer. At 14 days of culture, wells containing visible clones were scored positive. Pro-B cells were sorted from adult bone marrow as CD117+ CD19+ cells. All co-cultures were performed in Iscove's Modified Dulbecco's Medium (Sigma) supplemented with 2mM glutamine (Invitrogen), 0.5μM β-mercaptoethanol (Sigma) and 5% foetal calf serum (Labtech) in the presence of 1 ng/ml interleukin 7 (IL-7), 5 ng/ml fms-like tyrosine kinase-3 ligand (Flt3L) and 100ng/ml stem cell factor (SCF) (all bought from Peprotech).

Foetal Thymic Organ Cultures

Thymic lobes dissected from day 14.5 C57BL/6-Ly5.2 mouse embryos were treated with 1.35mM 2-deoxyguanosine (Sigma). Washed lobes were next reconstituted with 1 x 10^3 sorted Ly5.1 progenitor cells in a hanging drop and after 24 hours transferred to culture. At indicated time points, thymic lobes were harvested and mechanically disrupted to prepare single cell suspensions. Thymocytes were subsequently stained with indicated fluorochrome labelled antibodies. Donor derived cells were CD45.1+. All cultures were performed on 13mm Nucleopore® Track-Etch Membranes (Corning) in Iscove's Modified Dulbecco's Medium (Sigma) supplemented with 2mM glutamine (Invitrogen), 0.5μM β-mercaptoethanol (Sigma) and 10% foetal calf serum (Labtech) without cytokines.

In vivo Transplantation Assays

1 x 10^3 or 4 to 5 x 10^3 sorted progenitor populations from B6.EYFP mice were intravenously injected into *cd132-/-Rag-2-/-* hosts that had been given 250 rads 4-6 hours before. Mice were analysed for EYFP+ donor derived cells in bone marrow, thymus and spleen at indicated time points by flow cytometry. For the analysis of developmental potential in an athymic environment, 5 x 10^3 sorted progenitor populations from BALB/c *CD90.1* mice were intravenously injected into BALB/c *CD90.2* nude mice that received 250 rads 4-6 hours previously. Mice were analysed for CD90.1+ donor derived cells in bone marrow, mesenteric lymph nodes, peripheral lymph nodes and spleen 6 weeks after transplantation by flow cytometry.

Table 2.1 Primer Sequences and PCR Parameters Used in Semi-quantitative PCR.

PCR primers were designed and tested for secondary structure formation and primer dimerisation using the Primer 3 freeware. Primers were tested with total bone marrow or total double negative thymocyte cDNA to determine their specificity reflected in correct product size and optimal annealing temperature reflected in highest yield of product.

Gene	Size (bp)	Anneal (°C)	5' sequence (5'-3')	3' Sequence (5'-3')
pTα	449	63	GTGTCAGGCTCTACCATCAGG	GCAGAAGCAGTTTGAAGAGGA
GATA-3	285	63	TCGGCCATTCGTACATGGAA	GAGAGCCGTGGTGGATGGAC
Pax-5	439	63	CTACAGGCTCCGTGACGCAG	TCTCGGCCTGTGACAATAGG
PU.1	552	63	TGGAAGGGTTTTCCCTCACC	TGCTGTCCTTCATGTCGCCG
GM-CSFRα	264	63	GCGGGCGACACGAGGATGAAGCAC	CTAGGGCTGCAGGAGGTCCTTCCT
RelB	575	63	GATCCACATGGAATCGAGAG	AGATGTCCGATTCAGGATGA
Id2	405	63	ATGAAAGCCTTCAGTCCGGTG	TTAGCCACAGAGTACTTTGCT
IL-3Rα	181	58	TGCATGGACAACACTGTGGA	TGTATTCCTGTGGCTCTGAG
ICSBP	454	58	ATGTGTGACCGGAACGGCGG	CAGAAGGTTCCTTGATCAGC
HPRT	249	58	GCTGGTGAAAAGGACCTCT	CACAGGACTAGAACACCTGC
Notch-1	406	56	TGTAAGAATGCTGGAACGTG	GATAGGAGCCGATCTCATTGT
Flt-3	624	56	TCTTGAGACCGTTACAAACC	ATGTCTGTTCCGAACAACTC
IL-7Rα	304	56	CGAGTGAAATGCCTAACTC	GCGTCCAGTTGCTTTCAC

Table 2.2 Primer and Probe Sequences Used in Quantitative Real-time PCR.

Primer/probe sequences were manually designed using the QuantiTect Assay software available on the Qiagen website. Exon spanning sequences were chosen to ensure no signal derived from DNA contamination was registered. Primers were tested with titrated total bone marrow or titrated total double negative thymocyte cDNA by generating a regression curve of threshold cycle (CT) versus $\log_{10}$ template dilution. Correlation coefficients >0.98 meant a good primer probe combination.

Gene	Size (bp)	Anneal (°C)	5' sequence (5'-3')	Probe (5'-3')	3' Sequence (5'-3')
Notch-1	110	60	GAAGAACGGAGCCAACAA	GAACAACAAGGAGGAGA	GCAAAGTGGTCCAGCAACA
pTα	149	60	CAACTGGGTCATGCTTCT	GGCGTCAGGTGTCAGG	CTTCCATCTACCAGCAGTTGT
Hes-1	101	60	AGCCAACTGAAAACACTGAT	CCAAGCTAGAGAAGGCA	GAGGTGCTT*CACAGTCATT
Dtx-1	109	60	CAATGGCAACAAGGATGG	GG*GGAGAAGACAGG*GA	AGTGCGGGATGAGGTGAAA
GATA-3	100	60	TATCAAGCCCAAGCGAAG	AGACCACCACCACCAC	CATTAGCGTTCCTCCTCCAGA
Runx-1	129	60	GGTGGAGGTACTAGCTGA	CACCGACAGCCCCAAC	CAGTGCCACCACCTTGAAA
Rag-1	166	60	GCAGACATTCTAGCACTC	CCTCCTTGCCGTCTACC	GGATCTCACCCTAAACAGCTT
PU.1	124	60	CGGATGACTTGGTTACTTAC	GAGAAAGCCATAGCGA	GACATGGTGTGCGGAGAAAT
Arbp	77	60	TTTGACAACGGCAGCATT	CGACATCACAGAGCAGG	ACCCTCCAGAAAGCGAGA

Chapter 3

Phenotypic Analysis of Progenitor Thymocytes

Introduction

Expression of cell surface molecules has been utilised to phenotypically define distinct cellular populations. Based on these phenotypes, the functional attributes of cells could be established. Classically, double negative thymocytes have been classified by their lack of expression of CD4 and CD8 and various combinations of expression of CD44 and CD25 (Figure 1.3). More recently, an additional marker has been used to identify the earliest populations of double negative cells, the receptor tyrosine kinase c-kit (CD117). This molecule is expressed on adult and foetal haematopoietic stem and progenitor cells. DN1 cells were thus defined as $CD44^{+}CD117^{high}CD25^{-}$ and DN2 cells as $CD44^{+}CD117^{high}CD25^{+}$ (as reviewed by Rolink and Ceredig 2002).

An alternative strategy has been utilised to further subdivide the DN1 population based on expression of CD117 and CD24 (HSA, Heat Stable Antigen). CD24 is expressed on progenitor cells, pro-B cells and maturing T-cells. This approach yielded five distinct populations within the DN1 fraction, DN1a-e. DN1a exhibited high expression of CD117 and low/negative expression of CD24, DN1b was marked by high expression of CD117 and high expression levels of CD24, DN1c showed intermediate levels of CD117 expression but retaining high levels of CD24, whereas DN1d had no expression of CD117 and intermediate levels of CD24, and finally DN1e had lost expression of both of these molecules (Porritt, Rumfelt et al. 2004).

In our laboratory, a depletion strategy was devised, which minimally altered the composition of progenitor cells. Density gradient centrifugation was not implemented, since potentially, this may deplete certain fractions of cells. In addition, lineage depletion was achieved by introducing CD19 instead of CD45R to remove B-cells, because it has been shown that CD45R is expressed on lymphoid progenitors in the bone marrow (Martin, Aifantis et al 2003) and fractions of progenitor cells in the foetal

liver (Douagi, Colucci et al. 2002). CD4 was omitted from the depletion cocktail as well, because reports demonstrated expression of this antigen on early thymic progenitors (Wu, Scollay et al 1991).

With this strategy, the aim was to completely resolve double negative progenitors, in particular focusing on double negative 1 (DN1) population, since this population would harbour all prospective T-lineage progenitors, and to completely understand its composition therefore relating functional attributes of early thymic progenitors to their phenotype.

Results

Isolation of Double Negative Thymocytes

In order to obtain a pure population of double negative cells, mature T-cells were removed by depletion with CD8α and CD3ε. Since the thymus also harbours cells of other haematopoietic lineages, as illustrated by Figure 3.1B, these were removed by depletion with respective lineage markers, namely CD19 for B-cells, NK1.1 for natural killer cells, CD11b (Mac-1) for macrophages and granulocytes, and Ter119 for erythrocytes. CD4 and CD45R (B220) were not employed in the depletion strategy since these antigens are found on early thymic progenitors and the CLP-2 populations, respectively (Wu, Scollay et al. 1991; Martin, Aifantis et al. 2003). Ly-6G was also omitted since it is reportedly expressed on dendritic cell progenitors (Nakano, Yanagita et al. 2001). No density gradient was used and all steps were carried out at 4°C in order to minimise RNA degradation, which was important for subsequent experiments.

The optimised depletion procedure yielded pure double negative cells (Figure 3.2A), which can be further subdivided by CD44 and CD25 (Figure 3.2C and D). The composition of the DN compartment is as previously reported (Godfrey, Kennedy et al. 1993). The classical method of determining the percentages of various DN populations is by quadrant gating (Figure 3.2C); however, to generate more homogeneous cellular populations for sorting experiments and subsequent molecular and functional analyses, strictly defined regions were positioned (Figure 3.2D).

Figure 3.1 Composition of the Complete Adult Thymus.

A Representative histograms of total adult thymocytes from 4 weeks old female C57BL/6 mice stained for CD4/CD8, CD3ε and CD90.2 by flow cytometry. CD3ε was expressed at various levels, low, intermediate and high, according to the developmental stage. Homogeneous CD90.2 expression indicated a significant lack of contamination by stromal cells. Negative controls (black line) constitute isotype matched controls.

B Representative histograms illustrating expression of haematopoietic lineage markers gated on all viable thymocytes. CD19 marks B-cells, NK1.1 marks natural killer cells, CD11b marks macrophages and granulocytes, and Ter119 is expressed by mature erythrocytes and erythroid progenitors.

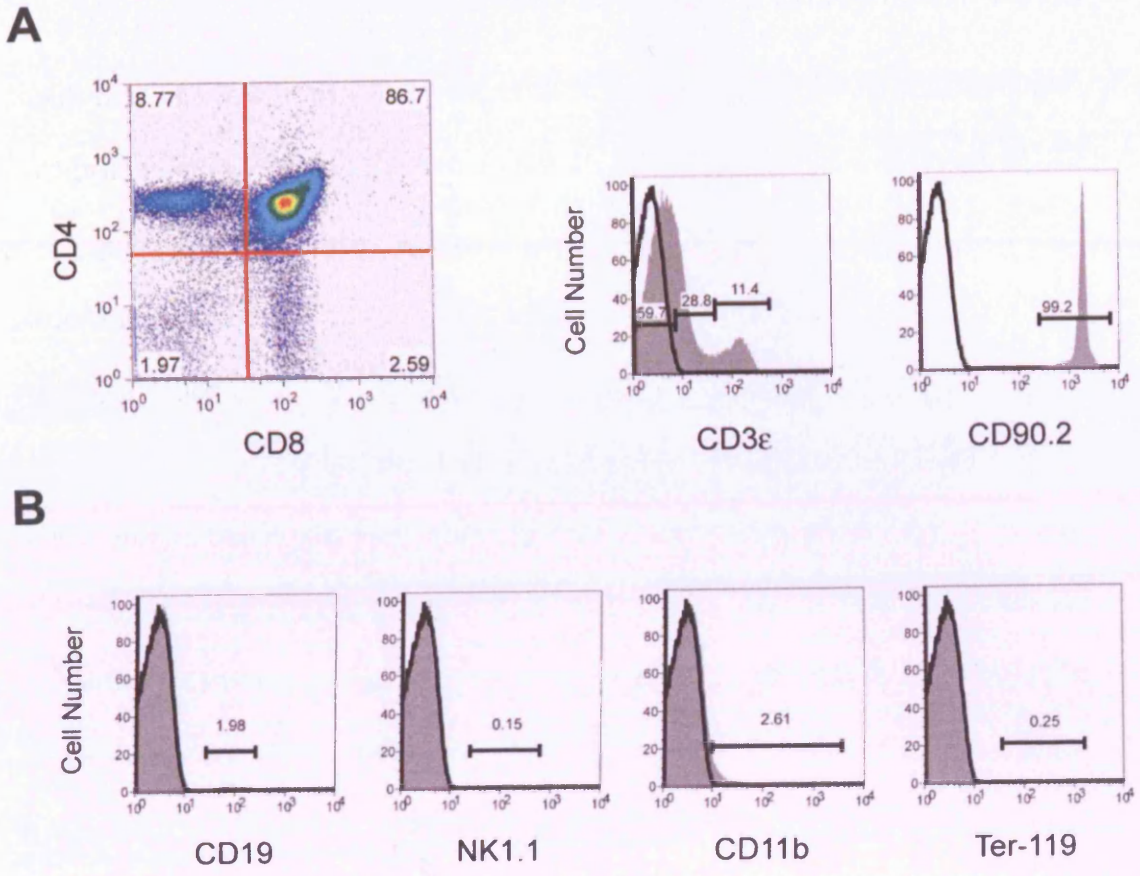

A
8.77
86.7
1.97
2.59
CD4
CD8
Cell Number
59.7
28.8
11.4
CD3ε
99.2
CD90.2
B
Cell Number
1.98
CD19
0.15
NK1.1
2.61
CD11b
0.25
Ter-119

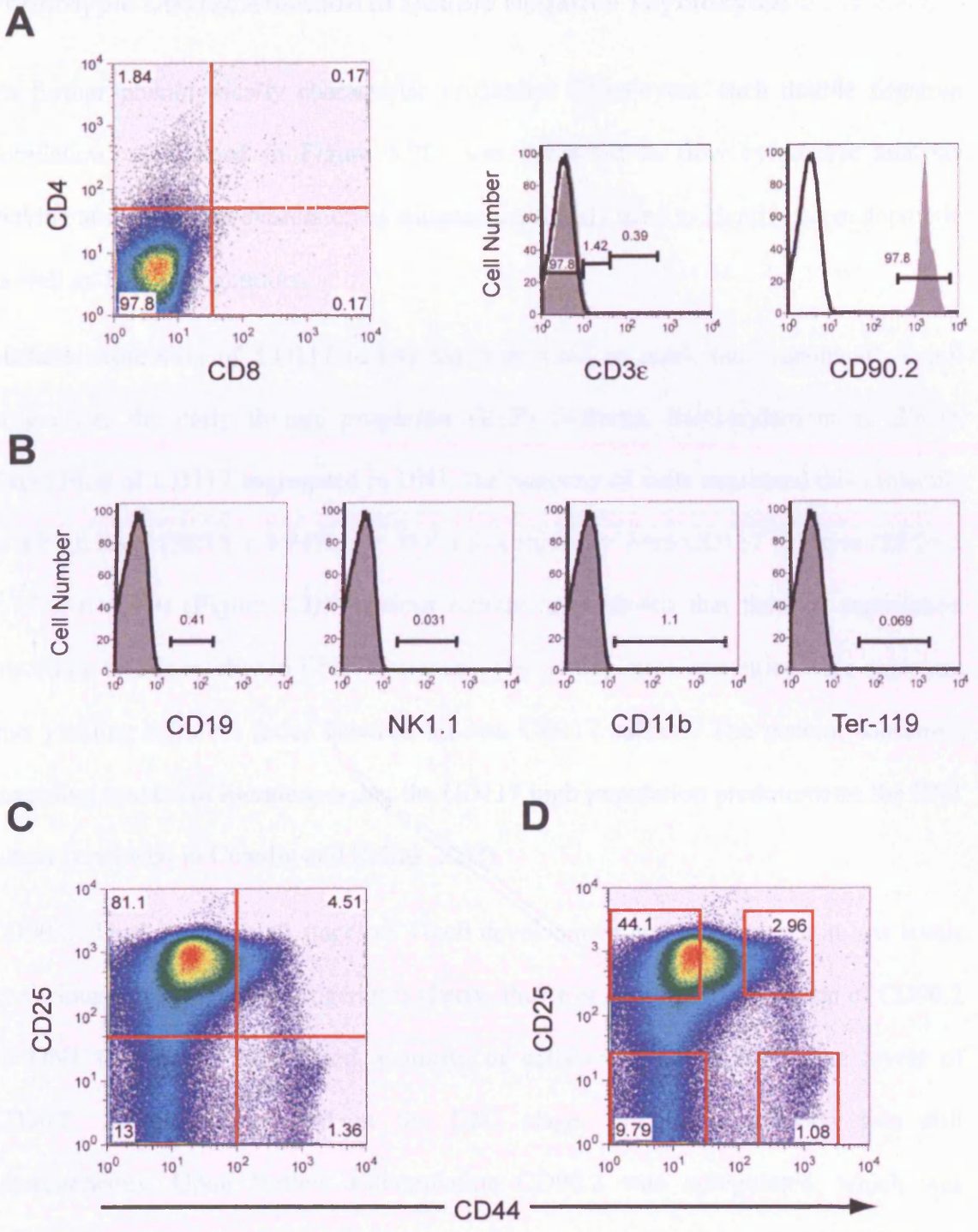
Figure 3.2 Composition of Double Negative Thymocytes.

A Representative histograms of the adult thymocytes from 4 weeks old female C57BL/6 mice after depletion of lineage positive cells. CD4/CD8 histogram illustrates efficient depletion procedure. Lack of mature T-cells is further corroborated by CD3ε expression. Most cells expressed T-cell lineage marker CD90.2.

B Representative histograms of total depleted thymocytes stained for lineage specific markers. Lack of any expression of lineage markers further illustrates an efficient depletion procedure.

C Representative histogram illustrating expression of CD44 and CD25 on lineage negative (double negative, DN) thymocytes. DN thymocytes segregated into four populations DN1 – DN4 by cell surface expression of CD44 and CD25, as defined by quadrant gating. DN1 are CD44+CD25-, DN2 are CD44+CD25+, DN3 are CD44-CD25+ and DN4 are CD44-CD25-.

D Representative histogram illustrating expression of CD44 and CD25 on DN thymocytes. Double negative populations are defined by strictly positioned gates, in order to achieve more homogeneous sub-sets. This gating approach was used for subsequent experiments.


A
1.84
0.17
97.8
0.17
CD4
CD8
Cell Number
97.8
1.42
0.39
CD3ε
97.8
CD90.2
B
Cell Number
0.41
CD19
0.031
NK1.1
1.1
CD11b
0.069
Ter-119
C
81.1
4.51
13
1.36
CD25
CD44
D
44.1
2.96
9.79
1.08
CD25
CD44

Phenotypic Characterisation of Double Negative Thymocytes

To further phenotypically characterise progenitor thymocytes, each double negative population, as defined in Figure 3.2D, was subjected to flow cytometric analysis looking at cell surface expression of antigens previously used to identify haematopoietic as well as T-cell progenitors.

Surface expression of CD117 (c-kit) has been used to mark the "canonical" T-cell progenitor, the early thymic progenitor (ETP) (Allman, Sambandam et al. 2003). Expression of CD117 segregated in DN1, the majority of cells expressed this molecule to a high level (58.13 ± 6.74%, n = 19)[1] and a minority were CD117 negative (28.26 ± 8.77%, n = 19) (Figure 3.3). Previous reports have shown that there is segregation according to this marker in DN1; however different depletion strategies were exploited thus yielding different ratios between the two CD117 subsets. The general consensus according to current literature is that the CD117 high population predominates the DN1 subset (reviewed in Ceredig and Rolink 2002).

CD90.2 (Thy1.2) marks all stages of T-cell development and is expressed at low levels by various haematopoietic progenitors (Perry, Pierce et al. 2003). Expression of CD90.2 on DN1 thymocytes segregated, majority of cells expressed intermediate levels of CD90.2. It was upregulated at the DN2 stage, but the expression was still heterogeneous. Upon further differentiation CD90.2 was upregulated, which was reflected by the increase in mean fluorescent intensity (MFI) (Figure 3.3). CD24 (Heat Stable Antigen, HSA) is expressed on early haematopoietic progenitors, pro-B-cells and maturing T-cells (Miller, Antognetti et al. 1985; Cooper, Mulvaney et al. 1986). DN1 thymocytes segregated into high and low CD24 expressing cells. From DN2 onwards

[1] Mean ± standard deviation, n = number of independent experiments.

the expression of CD24 was homogeneous, all cells expressed high levels of this cell surface antigen (Figure 3.3).

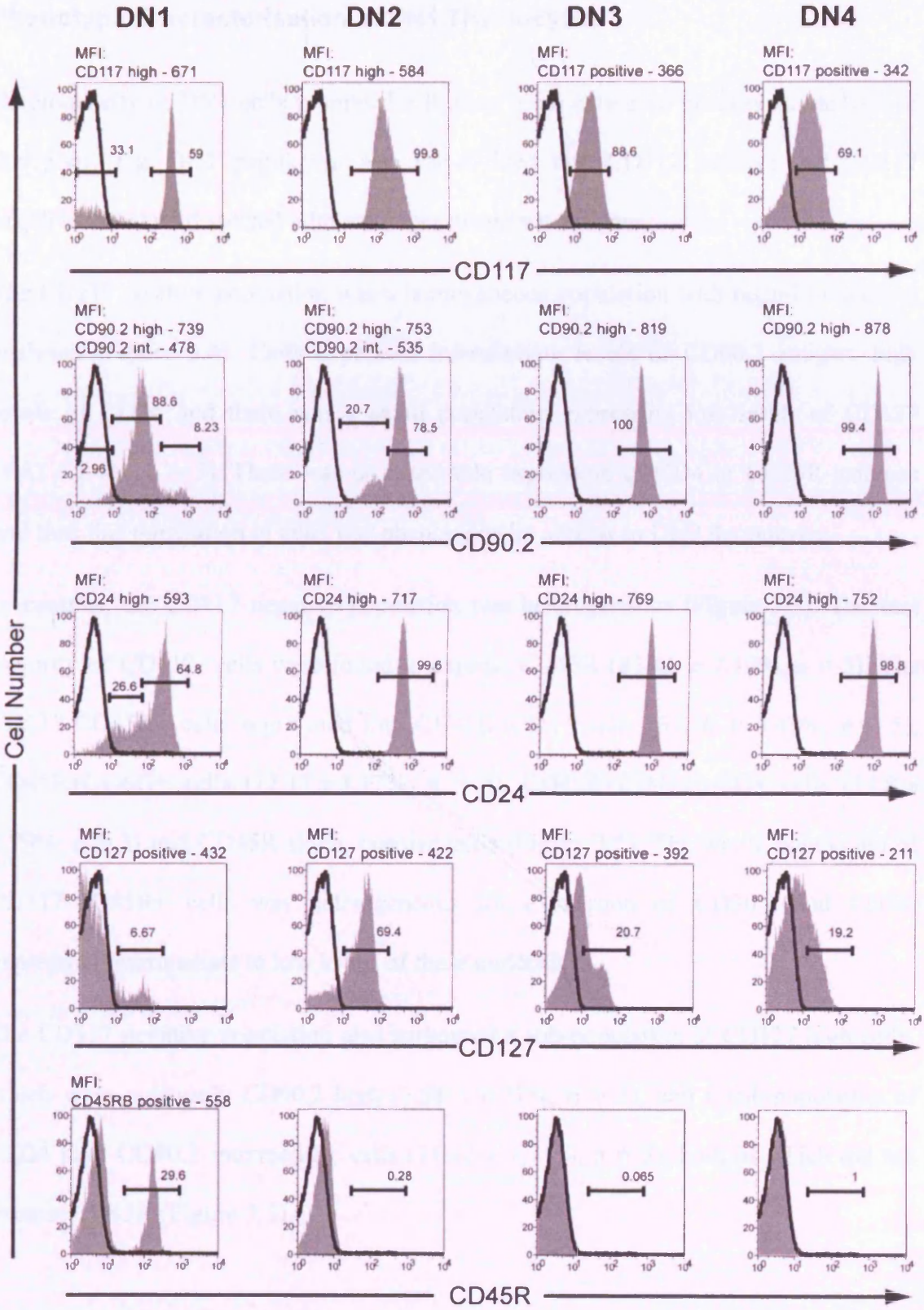
According to one model of haematopoietic development, cell surface expression of CD127 (interleukin 7 receptor α chain, IL-7Rα), marks commitment to the lymphoid lineage (Kondo, Weissman et al. 1997). Expression of CD127 was detected in only a small population of DN1 cells (Figure 3.3). This molecule was upregulated at the DN2 stage of development, but subsequently downmodulated as cells progressed through DN3 and DN4 stages.

Expression of CD45R (B220) is a hallmark of the common lymphoid progenitor 2 (CLP-2) population (Martin, Aifantis et al. 2003). Using CD19 instead of CD45R in the lineage depletion cocktail, a population expressing CD45R was detected in the DN1 fraction (23.59 ± 7.32%, n = 5) but was no longer evident at later stages of double negative development (Figure 3.3).

Figure 3.3 Phenotypic Analysis of Double Negative Thymocytes.

Representative histograms showing cell surface expression of CD117, CD90.2, CD24, CD127 and CD45R on double negative thymocytes form 4 weeks old female C57BL/6 mice, by flow cytometry. Expression of CD117 was heterogeneous in DN1, at DN2 expression was homogeneous, however intensity was reduced as compared to expressing cells in DN1, at DN3 and DN4 CD117 was downregulated. CD90.2 expression segregated in DN1 and was upregulated during subsequent stages of maturation, which was reflected in the mean fluorescent intensity (MFI). CD24 segregated in DN1, and was upregulated upon maturation. A small fraction of cells expressed CD127 in DN1, it was upregulated at DN2 and subsequently downmodulated. CD45R was expressed solely in the DN1 population, expression of this molecule was not seen at consequent stages of development. Expression of these cell surface antigens illustrates the heterogeneity of DN1 and to some extent, DN2 thymocytes. The negative controls (solid black line) constitute isotype matched controls.

MFI – mean fluorescent intensity.


DN1
DN2
DN3
DN4
MFI:
CD117 high - 671
33.1
59
MFI:
CD117 high - 584
99.8
MFI:
CD117 positive - 366
88.6
MFI:
CD117 positive - 342
69.1
CD117
MFI:
CD90.2 high - 739
CD90.2 int. - 478
88.6
8.23
2.96
MFI:
CD90.2 high - 753
CD90.2 int. - 535
20.7
78.5
MFI:
CD90.2 high - 819
100
MFI:
CD90.2 high - 878
99.4
CD90.2
MFI:
CD24 high - 593
26.6
64.8
MFI:
CD24 high - 717
99.6
MFI:
CD24 high - 769
100
MFI:
CD24 high - 752
98.8
CD24
MFI:
CD127 positive - 432
6.67
MFI:
CD127 positive - 422
69.4
MFI:
CD127 positive - 392
20.7
MFI:
CD127 positive - 211
19.2
CD127
MFI:
CD45RB positive - 558
29.6
0.28
0.065
1
CD45R
Cell Number

Phenotypic Characterisation of DN1 Thymocytes

Heterogeneity of DN1 cells prompted a further, more extensive phenotypic analysis of this pool. The DN1 population was sub-divided into CD117 positive and CD117 negative cells and dissected with a number of surface antigens.

The CD117 positive population was a homogeneous population with regard to markers analysed (Figure 3.4). Cells expressed intermediate levels of CD90.2 antigen, high levels of CD24 and there was a small population expressing low levels of CD127 (9.82 ± 1.4%, n = 5). There was no detectable expression of CD4 or CD45R antigens and thus this population of cells was phenotypically similar to DN2 thymocytes.

In contrast, the CD117 negative population was heterogeneous (Figure 3.5). The vast majority of CD117- cells were found to express CD45R (83.46 ± 7.10%, n = 5). The CD117-CD45R+ cells segregated into CD45R+CD4+ cells (65.16 ± 6.48%, n = 5), CD45R+Ly-6G+ cells (12.17 ± 1.17%, n = 3), CD45R+CD4+Ly-6G+ cells (14.8 ± 0.79%, n = 3) and CD45R single positive cells (Figure 3.5). The whole population of CD117-CD45R+ cells was heterogeneous for expression of CD90.2 and CD24, expressing intermediate to low levels of these molecules.

The CD117 negative population also harboured a sub-population of CD127 high cells, which were uniformly CD90.2 high (3.58 ± 0.71%, n = 5), and a sub-population of CD24 high CD90.2 intermediate cells (11.32 ± 2.71%, n = 5), both of which did not express CD45R (Figure 3.5).

Figure 3.4 Phenotypic Analysis of the DN1 CD117 Positive Thymocyte Population.

Representative histograms showing the gating strategy employed to resolve the DN1 CD117 positive population and its phenotype, from 4 weeks old female C57BL/6 mice. A strictly defined region was positioned on CD44+CD25- double negative thymocytes to define the DN1 population. This population was further dissected by the expression of CD117. Cells expressing high levels of CD117 were analysed for the expression of CD24, CD90.2, CD4, CD127 and CD45R. The DN1 CD117 positive population showed a homogeneous phenotype expressing intermediate levels of CD90.2 and mostly high levels of CD24. Only a small sub-set stained positive for CD127, whereas CD45R and CD4 were not expressed at all.

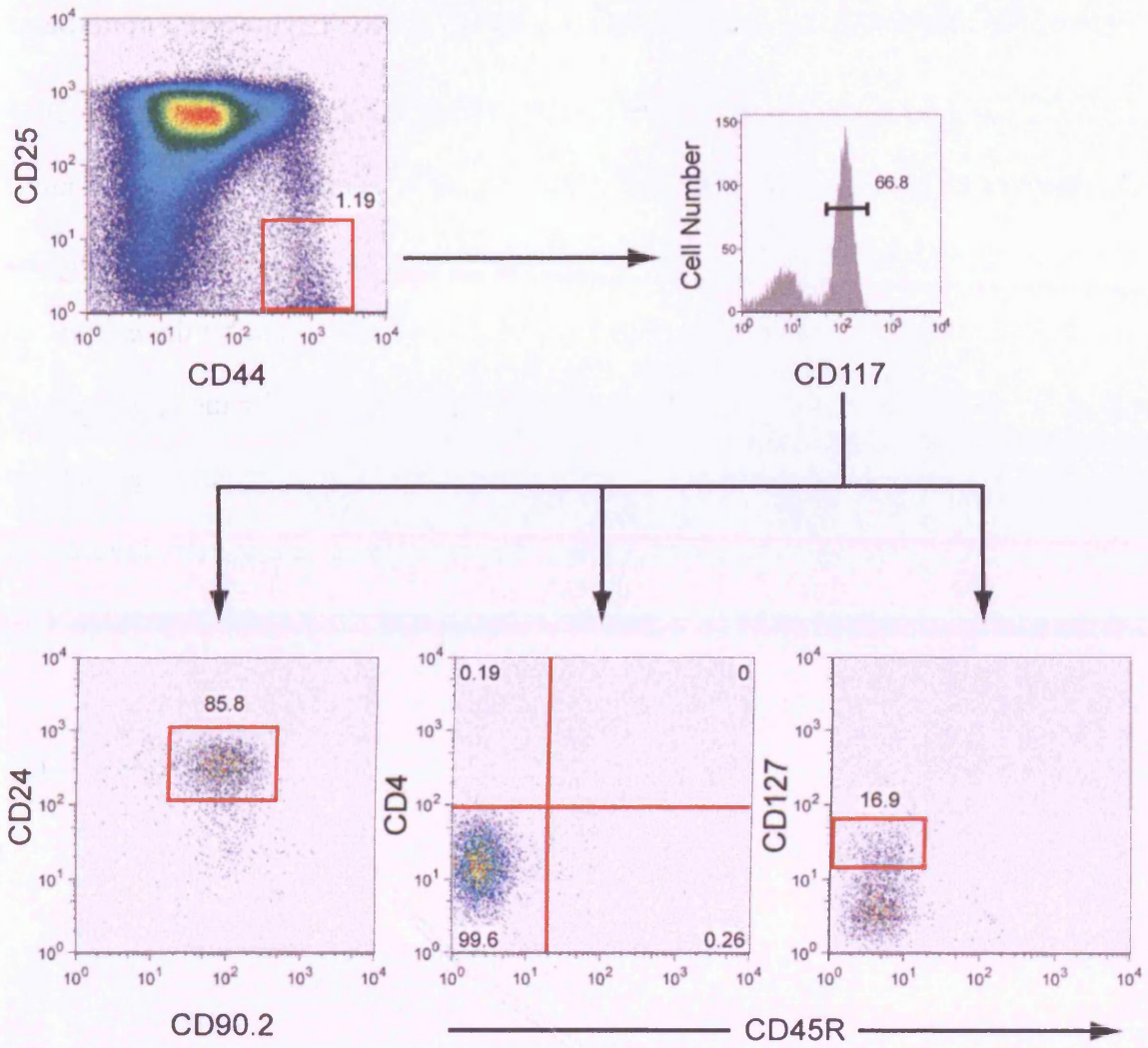
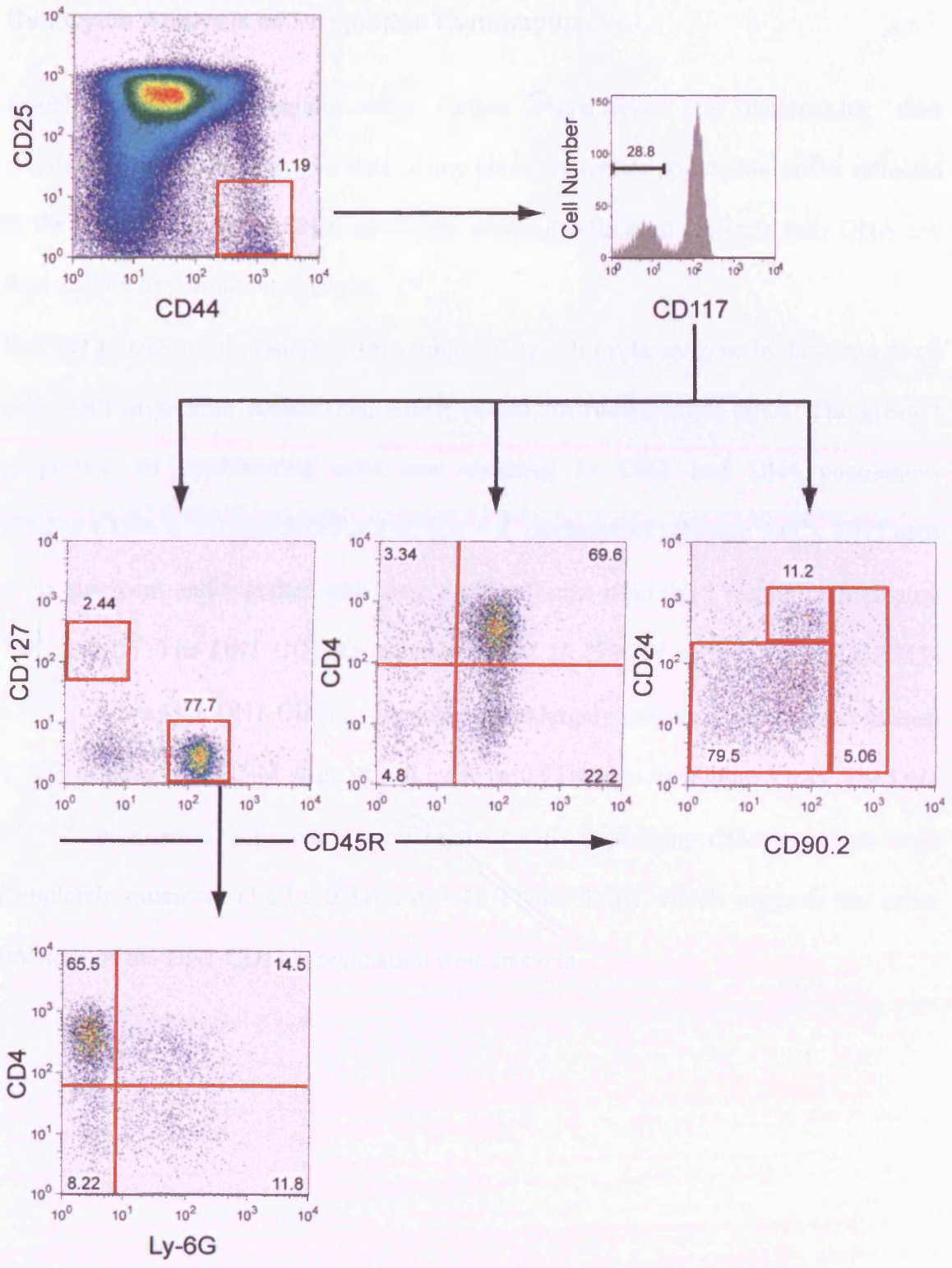

CD25
CD44
1.19
Cell Number
CD117
66.8
CD24
CD90.2
85.8
CD4
0.19
0
99.6
0.26
CD127
CD45R
16.9

Figure 3.5 Phenotypic Analysis of the DN1 CD117 Negative Thymocyte Population.

Representative histograms showing the gating strategy employed to resolve the DN1 CD117 negative population and its phenotype, from 4 weeks old female C57BL/6 mice. Strictly defined region was positioned on CD44+CD25- double negative thymocytes to define the DN1 population. This population was further dissected by the expression of CD117. Cells, which did not express CD117, were analysed for the expression of CD24, CD90.2, CD4, CD127, CD45R and Ly-6G. Representative histograms illustrate a heterogeneous phenotype of this population, segregating into CD45R positive and CD45R negative cells. The CD45R positive population is divided into four subsets: CD45R+CD4+, CD45R+Ly-6G+, CD45R+CD4+Ly-6G+ and CD45R single positive cells. CD45R negative population has two subsets, CD127 ^{high} CD90.2 ^{high} and CD24 ^{high} CD90.2 ^{intermediate} cells.


CD25
CD44
1.19
Cell Number
28.8
CD117
CD127
2.44
77.7
CD4
3.34
69.6
4.8
22.2
CD24
11.2
79.5
5.06
CD45R
CD90.2
CD4
65.5
14.5
8.22
11.8
Ly-6G

Cell Cycle Analysis of Progenitor Thymocytes

Double negative progenitors were further characterised by determining their proliferative state. Proliferative state of any given progenitor population can be reflected in the relative content of DNA, given that dividing cells must replicate their DNA and thus contain hyperdiploid amounts.

Purified progenitor thymocytes were subjected to cell cycle analysis by labelling fixed cells with propidium iodide (PI), which stoichiometrically binds DNA. The greatest proportion of proliferating cells was observed in DN2 and DN4 populations (35.5 ± 4.15% n = 2 and 50.95 ± 2.05% n = 2, respectively) (Figure 3.6C). DN3 cells were quiescent and together with their small cell size resembled resting cells (Figure 3.6C and D). The DN1 CD117+ population had 16.27% of cells in cycle, (± 2.71% n = 3), whereas the DN1 CD117- population was largely quiescent, however contained 5.77% of cells in S-G2-M stage of cell cycle (± 0.71%, n = 2) (Figure 3.6A). The DN1 CD117- population is primarily composed of cells expressing CD45R, which were completely quiescent (1.67 ± 0.44%, n = 2) (Figure 3.6B), which suggests that other fractions of the DN1 CD117- population were in cycle.

Figure 3.6 Cell Cycle Analysis of Double Negative Thymocytes.

Progenitor populations purified by FACS from 4 weeks old female C57BL/6 mice were fixed and stained with propidium iodide to analyse their DNA content. Representative histograms illustrate the frequency of cells in S-G2-M in each progenitor thymocyte population.

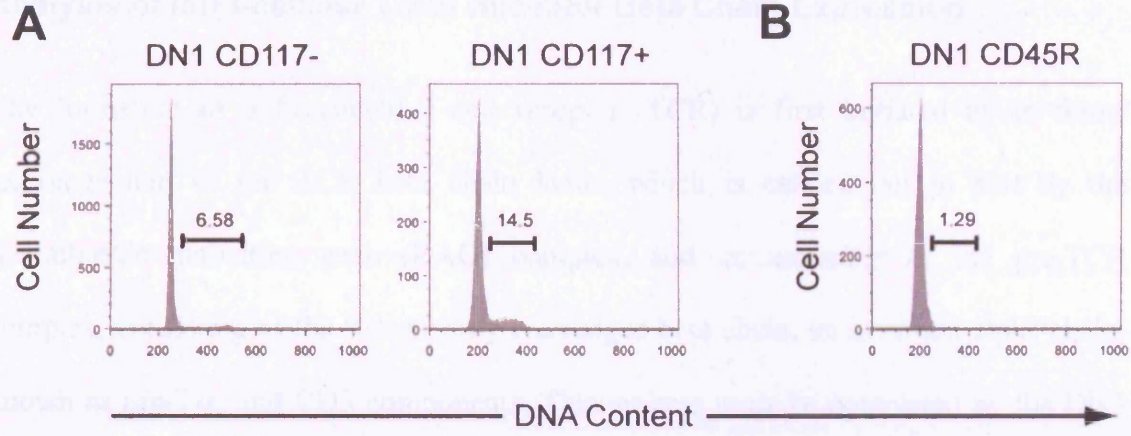
A Representative histograms showing the frequency of cell in S-G2-M in DN1 CD117 negative and positive progenitors. The DN1 CD117+ population has more cycling cells as compared to the DN1 CD117- population.

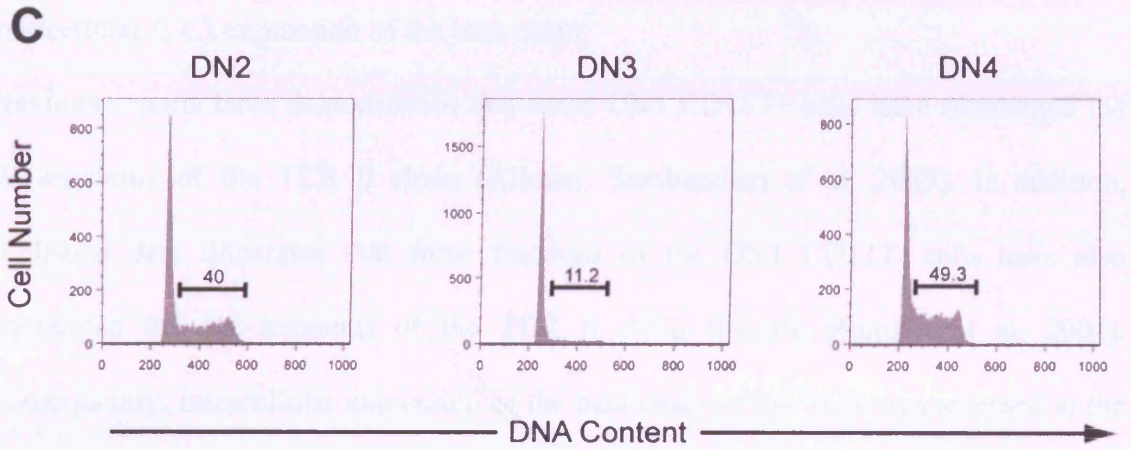
B Representative histogram illustrating the frequency of cycling cells in the DN1 CD117-CD45R+ population. This population is completely quiescent.

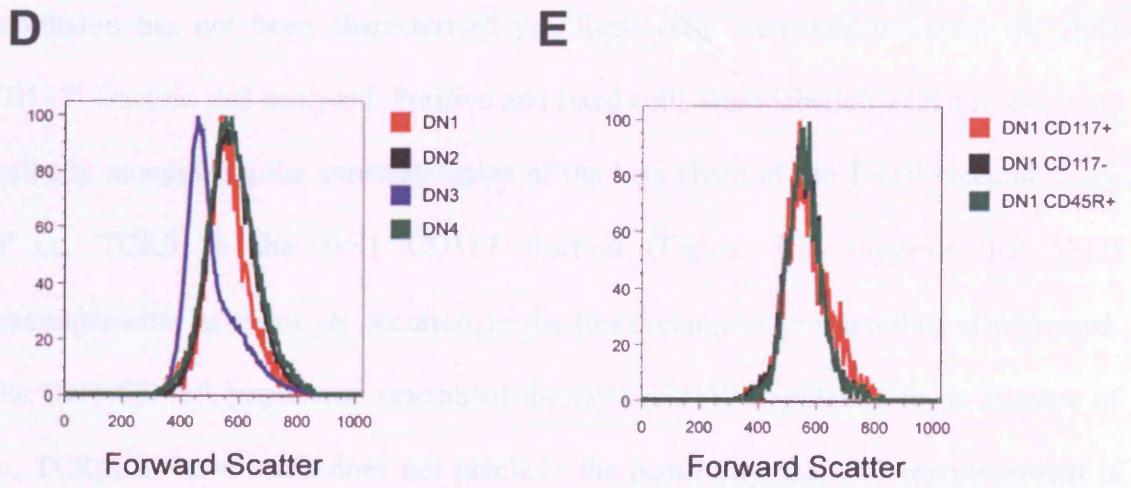
C Representative histograms depicting the frequency of cycling cells in the DN2, DN3 and DN4 progenitor populations. DN2 and DN4 populations contain large numbers of cycling cells, whereas DN3 is largely quiescent.

D Representative histograms illustrating the size of progenitor thymocytes as determined by forward scatter fluorescence. DN2 and DN4 cells are similar in size and are slightly larger than the DN1 population. The DN3 population is considerably smaller than the rest of double negative cells.

E Representative histograms illustrating the size of DN1 progenitors, as determined by forward scatter fluorescence. The CD117+ and CD117- cells are similar in size.


A
DN1 CD117-
DN1 CD117+
B
DN1 CD45R
Cell Number
6.58
14.5
1.29
DNA Content


C
DN2
DN3
DN4
Cell Number
40
11.2
49.3
DNA Content


D
E
DN1
DN2
DN3
DN4
DN1 CD117+
DN1 CD117-
DN1 CD45R+
Forward Scatter
Forward Scatter

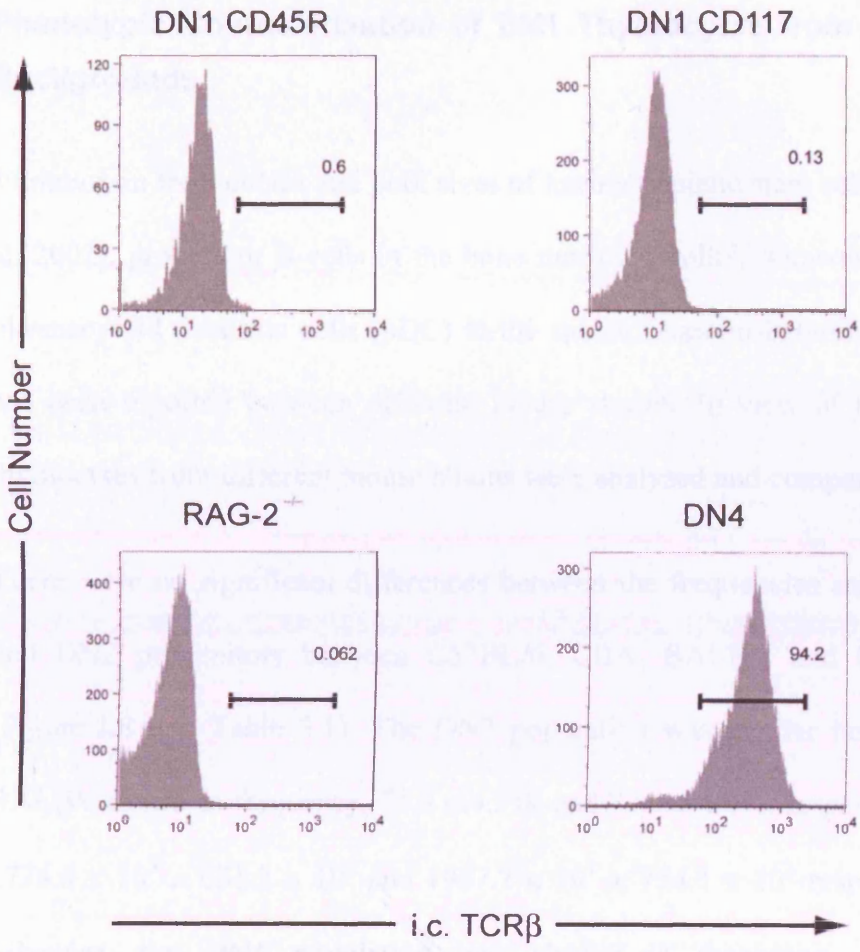
Analysis of Intra-cellular T-cell Receptor Beta Chain Expression

The formation of a functional T-cell receptor (TCR) is first initiated by in frame rearrangement of the TCR beta chain locus, which is carried out in part by the recombinase activating gene (RAG) complex, and an assembly of the pre-TCR complex, consisting of the successfully rearranged beta chain, an invariant alpha chain, known as pre-Tα, and CD3 components. This process must be completed by the DN3 stage if a cell is to progress to the DN4 stage, where all αβ T-cell precursors have intracellular (i.c.) expression of the beta chain.

Previous reports have demonstrated that some DN1 CD117+ cells have rearranged the DJ segments of the TCR β chain (Allman, Sambandam et al. 2003). In addition, published data illustrates that some fractions of the DN1 CD117- cells have also rearranged the DJ segments of the TCR β chain (Porritt, Rumfelt et al. 2004). Consequently, intracellular expression of the beta chain of the TCR was assessed at the protein level amongst the earliest progenitor thymocytes. Since the DN1 CD45R population has not been characterised yet, these cells were isolated from the DN1 CD117- fraction and analysed. Purified and fixed cells were labelled with a monoclonal antibody recognising the constant region of the beta chain of the T-cell receptor. Lack of i.c. TCRβ in the DN1 CD117 fraction (Figure 3.7) suggests that V-DJ rearrangements have not yet occurred, or the functional protein has not been expressed. The DN1 CD45R population resembled the DN1 CD117 population in its absence of i.c. TCRβ, however, this does not preclude the possibility that D-J rearrangement is taking place within the DN1 CD45R population.

Figure 3.7 Analysis of Intra-cellular T-cell Receptor Beta Chain Expression.

Representative histograms illustrating intra-cellular expression of the TCRβ chain in the DN1 thymocyte populations. Purified progenitor populations from 4 weeks old female C57BL/6 mice were fixed and stained intra-cellularly with an antibody recognising the C terminal constant domain of the T-cell receptor β chain. Both DN1 populations did not express the TCRβ chain at the protein level. *Rag-2* null mutants were used as a negative control, since no rearrangement occurs in the lymphoid cells of these mice, whereas DN4 cells served as a positive control.


DN1 CD45R
DN1 CD117
0.6
0.13
RAG-2 -/-
DN4
0.062
94.2
Cell Number
i.c. TCRβ

Phenotypic Characterisation of DN1 Thymocytes from Different Genetic Backgrounds

Variation in frequencies and pool sizes of haematopoietic stem cells (Morrison, Qian et al. 2002), progenitor B-cells in the bone marrow (Rolink, Grawunder et al. 1994) and plasmacytoid dendritic cells (pDC) in the spleen (Asselin-Paturel, Brizard et al. 2003) has been reported between different mouse strains. In view of this, double negative thymocytes from different mouse strains were analysed and compared.

There were no significant differences between the frequencies and pool sizes of DN1 and DN2 progenitors between C57BL/6, CBA, BALB/c and C3He mouse strains (Figure 3.8 and Table 3.1). The DN3 population was similar between C57BL/6 and BALB/c strains in frequency, 75.8 ± 4.5%, and 77.0 ± 4.0%, respectively, and numbers, 1774.4 x 10^3 ± 631.3 x 10^3 and 1957.7 x 10^3 ± 754.5 x 10^3 respectively (Table 3.1). Likewise, the DN4 population was similar in frequency, 17.8 ± 4.5% and 16.7% ± 4.5%, respectively, as well as the absolute cell number, 408.2 x 10^3 ± 162.7 x 10^3 and 391.3 x 10^3 ± 135.8 x 10^3 respectively. However, on the CBA background the DN3 population was less frequent as compared to the C57BL/6 strain, 65.5 ± 2.3%, $p < 0.01$, but more numerous as determined by the absolute cell number, 2681.3 x 10^3 ± 227.9 x 10^3, $p < 0.05$ (Table 3.1). The DN4 subset was more frequent, 29.1 ± 3.1%, $p < 0.01$ and consequently constituted a larger number of cells, 1181.3 x 10^3 ± 72.0 x 10^3, $p < 0.05$, in the CBA as compared to the C57BL/6 strain (Table 3.1).

The DN1 fraction of each mouse strain was next analysed and compared. Since the DN1 population can be conveniently subdivided into CD117+CD45R- (DN1 CD117) and CD117-CD45R+ (DN1 CD45R) cells, these markers were used to dissect the DN1

population of each mouse strain. As illustrated in Figure 3.8 the phenotypic composition of the DN1 population was consistent across all mouse strains analysed. However the frequencies and pool sizes of each progenitor pool fluctuated (Figure 3.8 and Table 3.1). The CD117 positive fraction predominated the DN1 population of C57BL/6 mice, 58.1 ± 6.7%, with the CD45R positive fraction constituting a smaller proportion, 23.6 ± 7.3%. All other strains exhibited an inverse relationship between CD117+ and CD45R+ cells (Figure 3.8 and Table 3.1). In CBA mice the CD117+ population comprised 17.4 ± 5.4%, whereas the CD45R+ fraction constituted 44.8 ± 8.2% of the total DN1 population. A similar observation was noted for the composition of the DN1 population in BALB/c and C3He mice (Table 3.1). Inverted frequencies were mirrored by the increase in absolute cell numbers of the DN1 CD45R population and a decrease in the DN1 CD117 population in CBA, BALB/c and C3He strains as compared to the C57BL/6 background (Table 3.1). Interestingly, there was a significant increase of cells in the DN1 population, which expressed CD127 in CBA, BALB/c and C3He mouse strains, as compared to the C57BL/6 background (Figure 3.8 and Table 3.1). The thymic analog to the common lymphoid progenitor (CLP) was detected in the thymus of all strains, but there was a significant increase of cells with this phenotype in the BALB/c background (Table 3.1). The CLP constituted 6.8 ± 1.0% and contained 3.0 x 10^3 ± 0.8 x 10^3 cells, which was significantly lager than the respective population on the C57BL/6 background (2.4 ± 0.4%, $p < 0.01$ and 1.0 x 10^3 ± 0.2 x 10^3 cells, $p < 0.01$).

Altogether, this illustrates that the C57BL/6 background is unlike all other commonly used experimental inbred mouse strains in the frequencies and pool sizes of the earliest progenitor thymocytes.

Figure 3.8 Composition of Double Negative Thymocytes from Different Genetic Backgrounds.

Representative histograms illustrating the composition of double negative thymocytes from 4 weeks old female C57BL/6 and CBA inbred mouse strains. Histograms depicting the composition of DN thymocytes as resolved by CD44 and CD25 expression, illustrate similar frequencies of DN1 population between the two mouse strains. Histograms in the shaded box represent the composition of CD44+CD25- DN1 thymocytes in the restricted gate, as resolved by CD117, CD45R and CD127 cell surface expression. The preponderant DN1 population in C57BL/6 mice expressed CD117, whereas in CBA inbred strain, cells expressing CD45R dominated the DN1 population. There was no significant difference in the frequency of the CD117 intermediate CD127 positive cells ("CLP") between these strains, whereas there was a significant increase in the frequency of CD127 positive CD117 negative cells in the CBA mouse strain, as compared to the C57BL/6 strain.

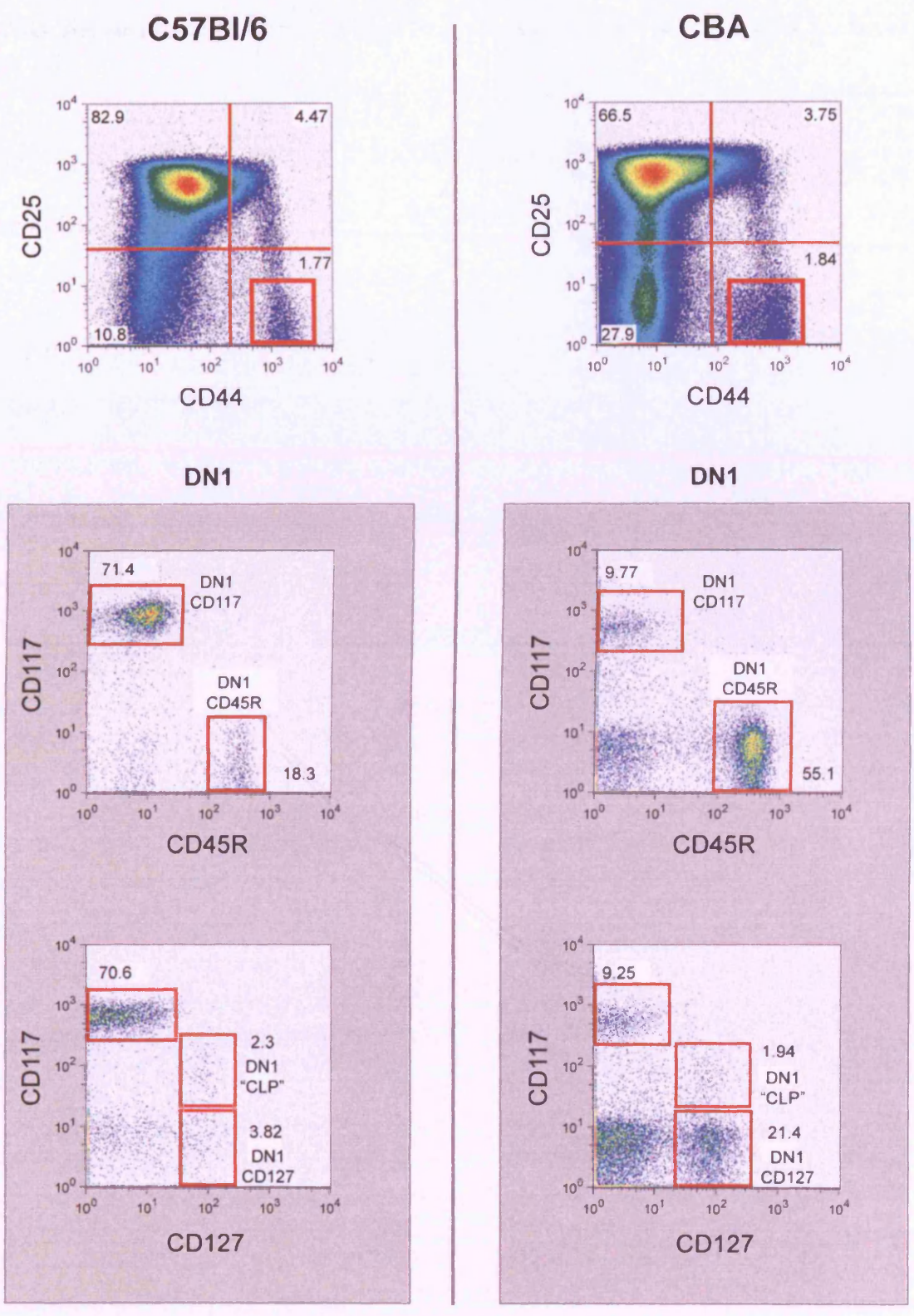

C57Bl/6
CBA
82.9
4.47
1.77
10.8
66.5
3.75
1.84
27.9
CD25
CD44
DN1
71.4
DN1
CD117
DN1
CD45R
18.3
9.77
55.1
CD117
CD45R
70.6
2.3
DN1
"CLP"
3.82
DN1
CD127
9.25
1.94
21.4
CD127

Table 3.1 Frequencies and Cell Numbers of Double Negative Sub-populations from Different Genetic Backgrounds.

Table representing frequencies in percentages and absolute cell numbers of double negative sub-populations from 4 weeks old female mice on the C57BL/6, CBA, BALB/c and C3He genetic backgrounds. Double negative thymocytes were resolved by cell surface expression of CD44 and CD25, frequencies and cell numbers were determined by the quadrant statistics as in Figure 3.8. The DN1 population was defined by a strictly positioned gate on the CD44+CD25- population, as in figure 3.2D, and further dissected by cell surface expression of CD117, CD45R and CD127. Each DN1 population corresponds to the respective population in Figure 3.8. For CBA and BALB/c mouse strains 3 independent experiments were used to calculate all parameters. For the C57BL/6 background, 19 independent experiments were used to calculate the composition of DN1-4 as well as the frequency and cell number of the DN1 CD117 population, whereas the percentages and cell numbers of DN1 CD45R, DN1 "CLP" and DN1 CD127 fractions were calculated from 5 independent experiments. Percentages of DN1-4 are expressed as percentages of total thymocytes. Percentages of various DN1 populations are expressed as percentages of total DN1. All values are mean ± standard deviation. Student's *t*-test was used to determine the significance of differences in frequencies and cell numbers between populations in C57BL/6 and all other strains.

* $p < 0.05$

** $p < 0.01$

	Frequency (%) (mean ± S.D.)				Absolute cell number (x 10^3) (mean ± S.D.)			
	C57BL/6	**CBA**	**BALB/c**	**C3He**	**C57BL/6**	**CBA**	**BALB/c**	**C3He**
DN1	1.7 ± 0.6	1.6 ± 0.2	1.9 ± 0.4	2.0	40.0 ± 19.4	63.3 ± 6.9	44.5 ± 10.0	65.4
DN2	4.7 ± 0.5	4.1 ± 0.7	4.4 ± 0.9	3.3	111.4 ± 41.7	166.0 ± 31.9	113.6 ± 56.9	109.6
DN3	75.8 ± 4.5	65.5** ± 2.3	77.0 ± 4.0	63.9	1774.4 ± 631.3	2681.3* ± 227.9	1957.7 ± 754.5	2110.1
DN4	17.8 ± 4.5	29.1** ± 3.1	16.7 ± 4.5	30.8	408.2 ± 162.7	1181.3** ± 72.0	391.3 ± 135.8	1017
DN1 CD117	58.1 ± 6.7	17.4** ± 5.4	18.3* ± 4.2	24	23.2 ± 11.6	10.7 ± 2.6	7.3* ± 1.3	15.7
DN1 CD45R	23.6 ± 7.3	44.8** ± 8.2	43.3* ± 13.0	43.9	9.3 ± 0.4	28.3* ± 7.6	20.4 ± 9.2	28.7
DN1 "CLP"	2.4 ± 0.4	3.1 ± 1.0	6.8** ± 1.0	11.6	1.0 ± 0.2	1.9 ± 0.4	3.0** ± 0.8	7.6
DN1 CD127	1.0 ± 0.7	18.4** ± 2.7	23.2** ± 6.7	17.4	0.4 ± 0.1	14.0* ± 4.6	9.9** ± 2.5	11.4

Phenotypic Analysis of Genetic Mutants

Genetic mutants have provided a wealth of information on functions of various gene products. In homozygous *Rag-2* null mutants the development of T-cells is arrested at the DN3 stage (Figure 3.9A). There are no mature T-cells, therefore allowing an easier access to the DN1 compartment, a population of particular interest.

A phenotypic analysis revealed that DN1 cells of *Rag-2* deficient mice on the C57BL/6 genetic background were not comparable in their composition to the C57BL/6 wild type DN1 cells (Figure 3.9B and C). DN1 cells in *Rag-2* null mutants did not clearly segregate into CD117 positive and negative cells; only 8.4% of DN1 cells expressed CD117 to a high level (± 6.6% n = 5), with the majority of cells expressing CD117 intermediate/low levels (Figure 3.9B). In wild type mice, majority of CD117 negative/low cells expressed CD45R (Figure 3.5), whereas only 5.6% of *Rag-2* null mutants expressed CD45R (± 1.0% n = 3). The population, which dominated the DN1 fraction of *Rag-2* deficient mutants, was the $CD127^{high}CD90.2^{high}$ population, comprising 79.2% (± 10.2%, n = 3) (Figure 3.9C), which in wild type animals is relatively minor. In contrast, DN2 and DN3 thymocytes were phenotypically similar to their wild type counterparts.

A similar developmental block was seen in mice carrying the $Lck^{-/-}Fyn^{-/-}$ double mutation on the C57BL/6 background (Figure 3.10A). The DN1 population of these mutants exhibited a similar phenotype to the DN1 population of *Rag-2* deficient mice, given that 60% of cells expressed CD117 at intermediate/low levels. As in the *Rag-2* null mutant, the more mature double negative populations showed a similar phenotype to their C57BL/6 wild type counterparts (Figure 3.10A).

Analysis of *cd127*$^{-/-}$ mutant on the C57BL/6 background revealed a block at the transition between DN2 and DN3 stages and a great reduction in cell numbers (Figure 3.10B and Table 3.2). An altered composition of DN1 thymocytes was observed, cells exhibiting CD117 intermediate phenotype. More mature DN cells were also phenotypically disparate to their wild type counterparts.

Figure 3.9 Phenotypic Analysis of *Rag-2* Null Double Negative Thymocytes.

A A representative histogram illustrating cell surface expression of CD44 and CD25 on double negative thymocytes from *Rag-2* null mutants on the C57BL/6 background from 4 weeks old mice. Total thymocytes were physically depleted of lineage positive cells and stained for surface antigens. Phenotypic analysis of *Rag-2* mutants revealed a complete block in T-cell development at the DN3 stage.

B Representative histograms depicting cell surface expression of CD117 and CD127 on double negative thymocytes as defined in **A**. The vast majority of DN1 cells expressed intermediate levels of CD117 and high levels of CD127. The negative control (black solid line) constitutes an isotype matched control.

C Representative histograms illustrating the composition of DN1 thymocytes in *Rag-2* null mutants as gated in **A**, resolved by cell surface expression of CD117, CD45R, CD90.2 and CD127. The vast majority were CD127 high CD90.2 high.

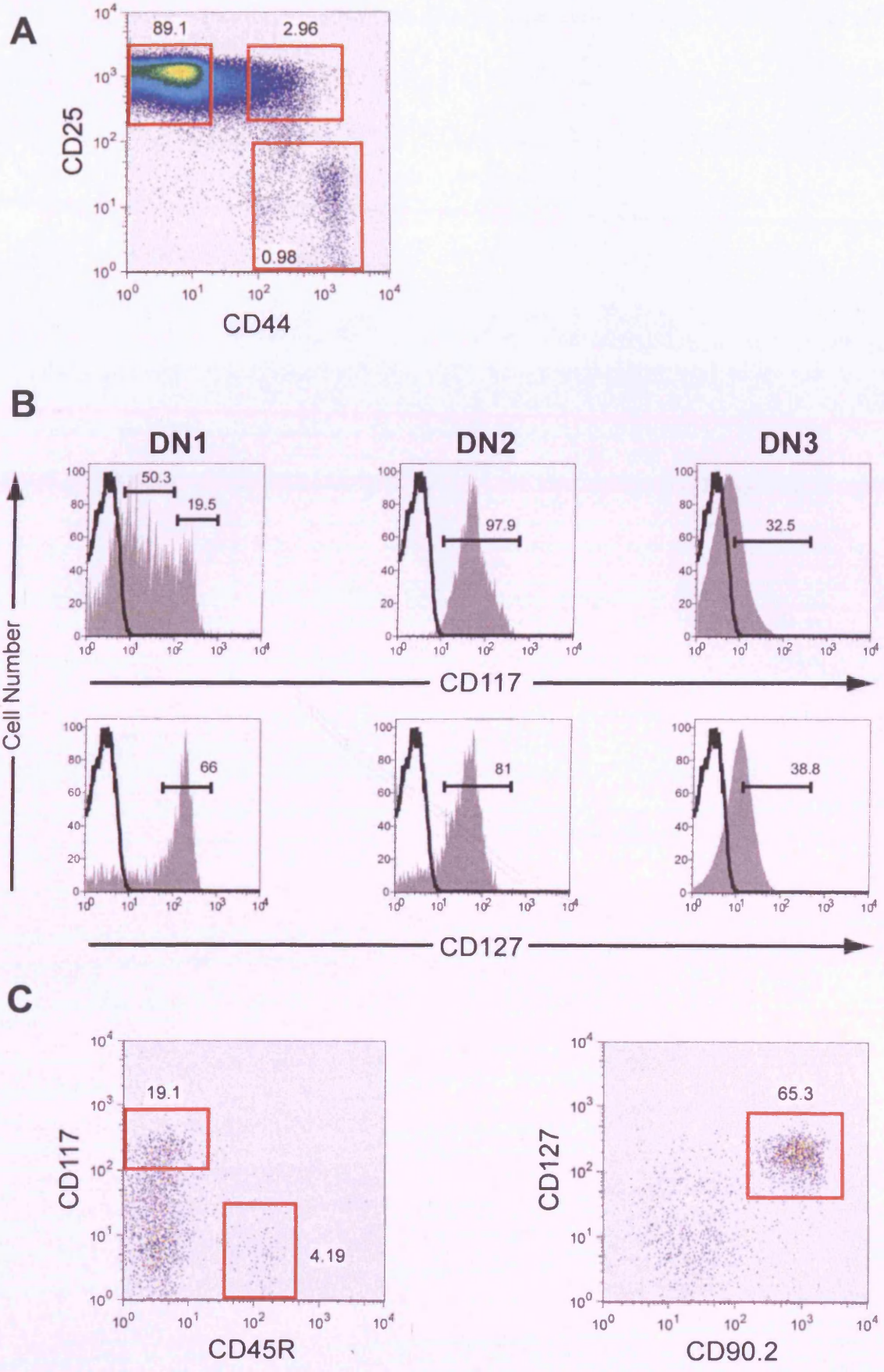

A
89.1
2.96
0.98
CD25
CD44
B
DN1
DN2
DN3
50.3
19.5
97.9
32.5
66
81
38.8
Cell Number
CD117
CD127
C
19.1
4.19
65.3
CD117
CD45R
CD127
CD90.2

Figure 3.10 Phenotypic Analysis of Double Negative Thymocytes in Genetic Mutants.

A Phenotypic analysis of *Lck/Fyn* double mutant on the C57BL/6 background from 4-6 weeks old mice. Total thymocytes were physically depleted of lineage positive cells and stained for CD44, CD25 and CD117. Flow cytometric analysis revealed a complete block in T-cell development at the DN3 stage as exhibited by CD44 and CD25 cell surface expression. DN1 cells expressed intermediate to low levels of CD117. The negative control (black solid line) constitutes an isotype matched control.

B Phenotypic analysis of *IL-7* mutant on the C57BL/6 background from 4-8 weeks old mice. Total thymocytes were physically depleted of lineage positive cells and stained for CD44, CD25 and CD117. Flow cytometric analysis revealed a partial block in T-cell development at the transition between DN2 and DN3 stage. DN1 population expressed CD117 to an intermediate level. The negative control (black solid line) constitutes an isotype matched control.

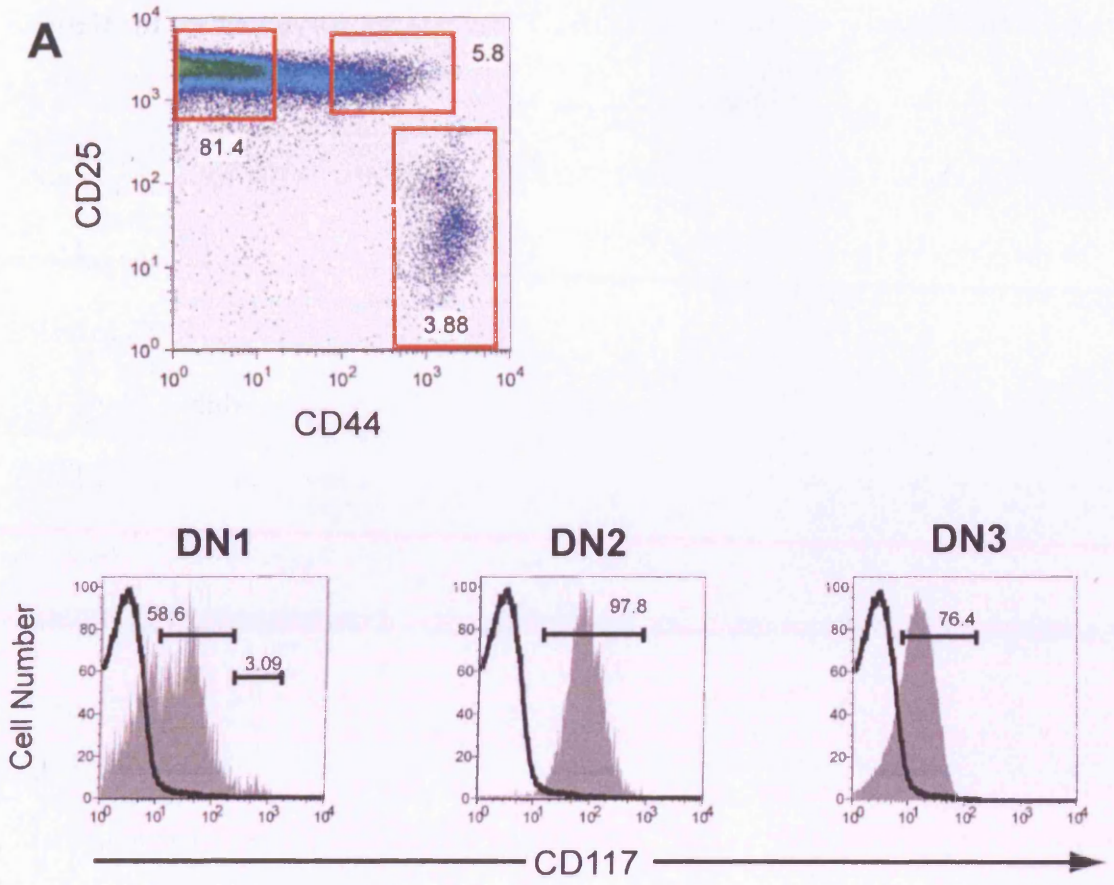

A
CD25
CD44
81.4
5.8
3.88
DN1
DN2
DN3
Cell Number
58.6
3.09
97.8
76.4
CD117
B
CD25
CD44
48.9
31.8
DN1
DN2
Cell Number
64.1
27.4
44.3
0.76
CD117

Table 3.2 Cell Numbers of Double Negative Thymocytes from Genetic Mutants.

A table of absolute cell numbers of double negative thymocyte sub-populations per adult thymus in wild type, *Rag-2*, *Lck/Fyn* and *IL-7* null mutants, all on the C57BL/6 genetic background.

	C57BL/6 Cell number (x 10^3)	RAG2$^{-/-}$ Cell number (x 10^3)	Lck/Fyn$^{-/-}$ Cell number (x 10^3)	IL-7$^{-/-}$ Cell number (x 10^3)
DN1	40.0	6.0	6.5	0.12
DN2	111.4	23.6	8.3	0.25
DN3	1774.4	720.0	151.0	N.D.

Alternative Definitions of Early Progenitor Thymocytes

A number of strategies have been employed in defining and dissecting the earliest populations of double negative cells (Balciunaite, Ceredig et al. 2005; Porritt, Rumfelt et al. 2004). DN1 cells have been classically defined as CD44+CD25-, however, an alternative gating scheme has been utilised to identify DN1 cells. Initially, high expressors of CD117 and CD44 are gated on and further subdivided into DN1 and DN2 based on expression of CD25 (Figure 3.11A). Thus the DN1 cells are classed as CD44+CD117^{high}CD25- and the DN2 population as CD44+CD117^{high}CD25+ and are here referred to as "alternative" DN1 and "alternative" DN2. At this point, this gating strategy was employed and compared with the conventional approach to characterise the DN1 and the DN2 populations.

The "alternative" DN1 population exhibited expression of CD90.2 to an intermediate level, high expression of CD24, a complete lack of CD45R expression and a small population expressed CD127 (Figure 3.11B). This phenotype is consistent with the published data (Balciunaite, Ceredig et al. 2005) and closely resembled the conventional DN1 CD117 population. The "alternative" DN2 population demonstrated high expression of CD24, segregation in expression of CD127 and a segregation of CD90.2 expression (Figure 3.11B). This is a similar phenotype to the conventional DN2 population, however, the ratio of CD90.2 intermediate to CD90.2 high cells was altered (Figure 3.11B). Conventional approach lead to a 1:4 ratio of DN2 CD90.2 intermediate to DN2 CD90.2 high cells (Figure 3.5), whereas the alternative approach yielded a 1:1 ratio (Figure 3.11B), which illustrates a bias toward the immature DN2 population. An overall reduction in total DN1 and DN2 numbers was also observed (Table 3.3).

Expression of CD117 and CD24 has been used to dissect the DN1 population as defined by CD44 and CD25 cell surface expression (Porritt, Rumfelt et al. 2004). This approach resolved five populations, DN1a-e, and is illustrated in Figure 3.11C. The composition of the DN1 fraction based on these phenotypic markers is in line with published observations (Porritt, Rumfelt et al. 2004). However, the frequency and cell number of each respective population disagrees from the published data (Figure 3.11C, Table 3.3). Population DN1e predominated the DN1 fraction as described by Porritt *et al.* and comprised 54.8 ± 16% (n = 4). The DN1e population as illustrated in Figure 3.10B constituted only 17.43 ± 3.29% (n = 3), whereas the principal DN1 population in our study was the DN1b population 50.5 ± 4.9% (n = 3) (Figure 3.11C). The DN1b population in the study of Porritt *et al.* made up 11.2 ± 2.7% (n = 4) of the total DN1 pool. This illustrates that additional steps in preparation of double negative thymocytes, such as the density gradient centrifugation implemented in the study of Porritt *et al.*, and unconventional definitions of early double negative cells, greatly affects composition of the populations under investigation.

Figure 3.11 Alternative Definitions of Early T-cell Progenitors.

A A representative histogram illustrating cell surface expression of CD44 and CD117 on double negative thymocytes from 4 weeks old female C57BL/6 mice. The histogram depicts another method in identifying early T–cell progenitors based on high expression of CD44 and high expression of CD117 (Balciunaite, Ceredig et al. 2005).

B Representative histograms showing expression of CD24, CD90.2, CD45R and CD127 on DN1 and DN2 thymocytes as gated on CD44 high CD117 high cells in **A**. DN1 are CD25 negative, DN2 are CD25 high.

C Representative histogram depicting the classification of DN1 thymocytes based on expression of CD117 and CD24. DN1 thymocytes are CD44+CD25- and resolve into 5 populations, DN1a-e. This gating strategy was taken from Porritt *et al.* (Porritt, Rumfelt et al. 2004).

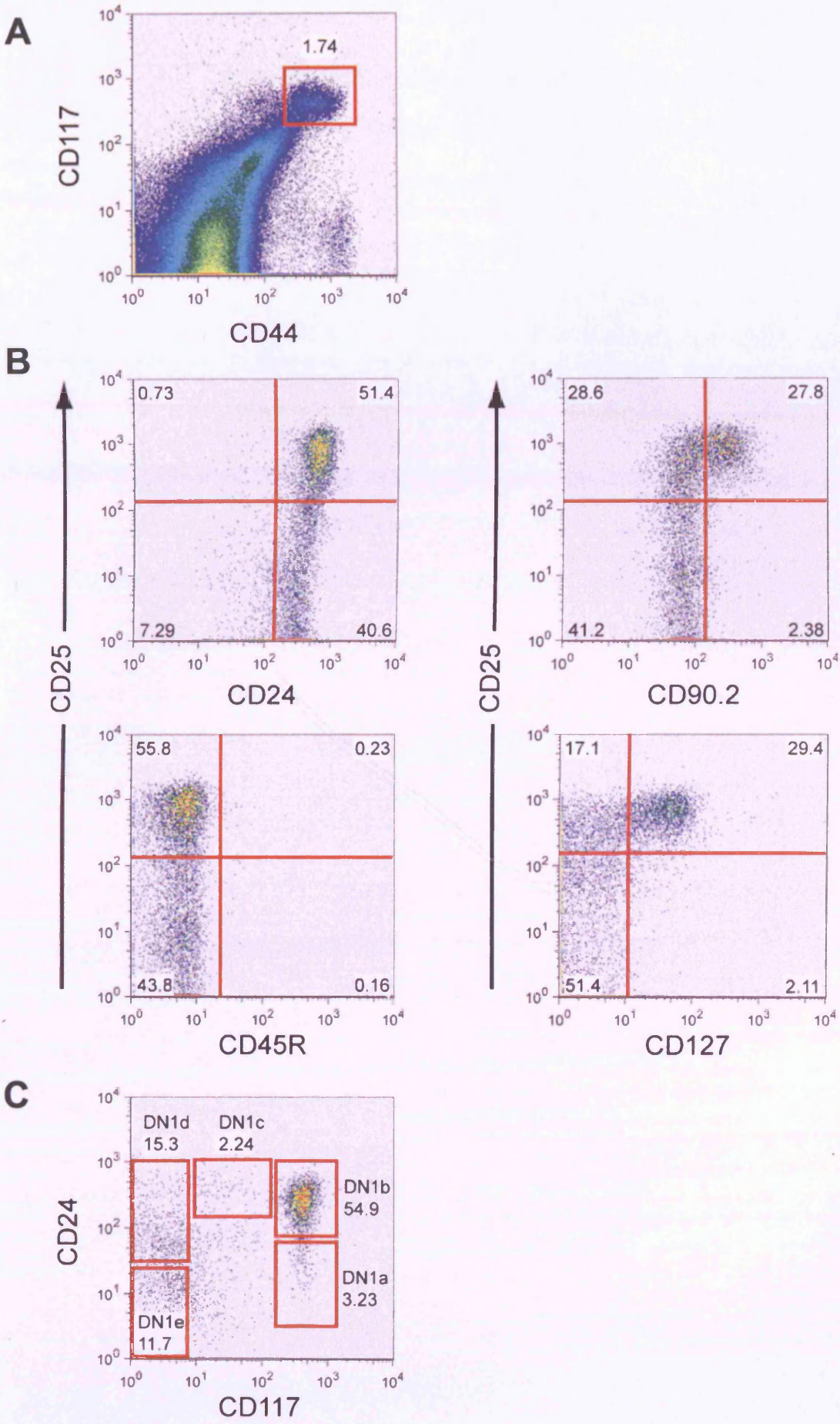

A
1.74
CD117
CD44
B
0.73
51.4
7.29
40.6
CD25
CD24
28.6
27.8
41.2
2.38
CD90.2
55.8
0.23
43.8
0.16
CD45R
17.1
29.4
51.4
2.11
CD127
C
DN1d
15.3
DN1c
2.24
DN1b
54.9
DN1a
3.23
DN1e
11.7
CD24
CD117

Table 3.3 Frequencies and Cell Numbers of Double Negative Populations as Defined by Alternative Gating Approaches.

Frequencies and cellularities of double negative progenitors defined by alternative gating strategies as depicted in Figure 3.11. "Alternative" DN1 was defined as CD44+CD117+CD25- and "alternative" DN2 was defined as CD44+CD117+CD25+. DN1a-e were resolved by cell surface expression of CD117 and CD24 gated on CD44+CD25- double negative thymocytes. Percentages of DN1 and DN2 populations are expressed as a percentage of total double negative thymocytes. Percentages of DN1a-e are percentages within total DN1 population. Cellularity as calculated by Porritt *et al.*, assumes that double negative population comprises 5% of total thymocytes, DN1 population constitutes 2% of total double negative thymocytes and the cellularity of the complete thymus is 2.5 x 10^8 cells (Porritt, Rumfelt et al. 2004). Student's *t*-test was used to determine the significance of differences in frequencies and cell numbers between populations as gated by the conventional or alternative methods.

** $p < 0.01$

*** $p < 0.001$

	Conventional		Alternative		Porritt et al.	
	Frequency (%) (Av. ± S.D.)	Cell number (x 10^3) (Av. ± S.D.)	Frequency (%) (Av. ± S.D	Cell number (x 10^3) (Av. ± S.D.)	Frequency (%) (Av. ± S.D.)	Cell number (x 10^3) (Av. ± S.D.)
DN1	1.7 ± 0.6	40.0 ± 19.4	0.9*** ± 0.3	20.2** ± 9.7		
DN2	4.7 ± 0.5	111.4 ± 41.7	1.3*** ± 0.5	29.6*** ± 14.7		
DN1a	3.6 ± 0.4	1.6 ± 0.6			1.4 ± 4.90	3.5 ± 4.90
DN1 b	50.5 ± 4.9	22.2 ± 12.3			11.2 ± 4.90	28 ± 4.90
DN1 c	2.2 ± 0.4	1.00 ± 0..2			14.2 ± 4.90	36 ± 4.90
DN1 d	17.3 ± 2.2	7.6 ± 1.1			18.5 ± 4.90	46 ± 4.90
DN1 e	17.4 ± 3.3	7.7 ± 1.2			54.8 ± 4.90	137 ± 4.90

Discussion

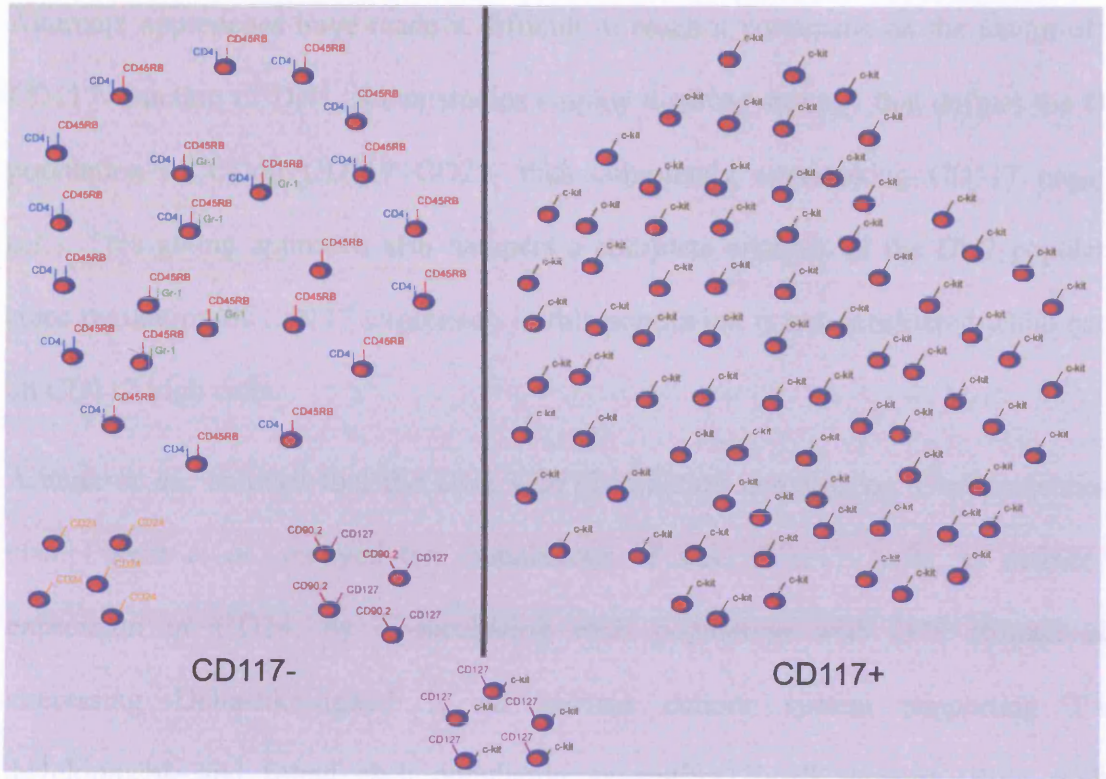
Many studies have focused on functional and phenotypic characterisation of DN1 thymocytes and have reported heterogeneity on both levels. DN1 cells are able to generate a number of haematopoietic lineages and harbour various subsets as defined by surface antigens (reviewed by Ceredig and Rolink 2002). However, isolation protocols vary from group to group thus altering cellular representation of different compartments in the DN1 population. This study provides a thorough phenotypic analysis of this population with minimal alterations to its cellular composition due to the preparative strategy employed. The density gradient centrifugation was not used, and in addition the lineage depletion cocktail did not contain CD4 nor CD45R, since these molecule have been shown to be expressed on early thymic and bone marrow progenitors, respectively (Wu, Scollay et al. 1991; Martin, Aifantis et al. 2003). Figure 3.12 illustrates the make up of the DN1 population.

The early thymic progenitor (ETP) has been branded as the "canonical" T-cell progenitor (Allman, Sambandam et al. 2003). These cells were able to generate T- as well as B-cells in a functional transplantation assay, and a residual myeloid potential was demonstrated in an *in vitro* culture system. The ETPs had detectable rearrangements at the T-cell as well as B-cell receptor loci and were phenotypically described as DN1 CD117 positive cells. The multipotent nature of DN1 CD117+ cells could arise from the possibility that distinct progenitor populations with specific functional capabilities coexist within this pool. This functional heterogeneity might be reflected in a heterogeneous phenotype of these cells. Phenotypic characterisation of this fraction of DN1 cells presented here is in perfect agreement with previously published data and illustrates that the DN1 CD117+ population is phenotypically homogeneous closely resembling DN2 cells, thus implying that at the population level

DN1 CD117+ cells are multipotent. Processes taking place in DN1 CD117+ cells, such as the upregulation of CD127 (interleukin 7 receptor α chain, IL-7Rα) underscore the idea of a precursor product relationship between these two populations.

Figure 3.12 Cellular Compartments of DN1 Progenitor Thymocytes.

The diagram represents the cellular composition of the DN1 population of adult murine thymocytes from the C57BL/6 mouse strain. The cells segregate into two main fractions as determined by CD117 expression. The CD117+ population is the preponderant population on the C57BL/6 background and expresses high levels of CD24 and intermediate levels of CD90.2. There is no expression of CD45R or CD127 in the DN1 CD117+ population. The CD117- cell population is heterogeneous and contains a majority that express CD45R. The CD117-CD45R+ population is itself heterogeneous, some cells express Ly-6G, some cells express Ly-6G and CD4, and some cells express neither molecule, whereas most cells express only CD4. On the whole, the CD117-CD45R+ population exhibits heterogeneous levels of CD24 and CD90.2 expression, but no expression of CD127. There is a small population within the CD117- fraction that concomitantly express high levels of CD127 and CD90.2. In addition, the CD117- population harbours a small population that exclusively expresses CD24. The DN1 population also contains a fraction of cells that express intermediate levels of CD117 and high levels of CD127, and by this cell surface phenotype resemble the common lymphoid progenitor population identified in the bone marrow.


CD117-
CD117+

Alternate approaches have made it difficult to reach a consensus on the nature of the CD117- fraction of DN1. Some studies employ a gating strategy that defines the DN1 population as CD44+CD117+CD25- thus completely overlooking CD117 negative cells. This gating approach also hampers a complete analysis of the DN2 population since regulation of CD117 expression in this population is not considered while gating on CD117 high cells.

Allman *et al.,* showed that the DN1 CD117- fraction contains no T-cell potential *in vivo*. Porritt *et al.,* assayed two populations of DN1 CD117- cells, as defined by expression of CD24, by co-incubating each population with OP9 stromal cells expressing Delta-like-ligand 1, an *in-vitro* culture system supporting T-cell development, and found each population generating T-cell progeny. Both studies, however, overlooked the majority of DN1 CD117- cells in our preparation, since CD45R was used in their depletion procedures. As illustrated here, DN1 CD117- population contains a preponderant and heterogeneous subset of CD45R+ cells, which showed no any characteristics of B-cells and might harbour some T-cell potential. A population of CD45R+ cells has previously been described in the bone marrow based on cell surface expression of human CD25 driven by the regulatory elements of murine *pre-Tα* gene (Gounari, Aifantis et al. 2002). These cells efficiently migrated to the thymus and generated T–cells (Scimone, Aifantis et al. 2006). However, analysis of transgene expression in the thymus revealed that only a small proportion of CD45R+ cells was marked by this reporter further underlining the fact that this population of cells is heterogeneous. Alternatively, variegation of the transgene expression could have resulted in such an outcome. The other two subsets of DN1 CD117- population shown here, the $CD24^{high}CD90.2^{intermediate}$ and the $CD127^{high}CD90.2^{high}$ populations, are phenotypically indistinguishable from populations described by Porritt *et al.*, who

showed that the latter population has already rearranged the TCRβ locus. The $CD127^{high}CD90.2^{high}$ population phenotypically resembles DN2 cells, and is the predominant DN1 population of the *Rag-2* null mutant, where DN2 and DN3 cells are phenotypically comparable to wild type pools. The phenotype and the exclusive presence of these cells in a mutant imply a role of CD127 positive cells as an alternative intermediate in T-cell development. The discrepancy observed between the study of Porritt *et al.*, and results presented here on the relative composition of the DN1 population might arise from an alternative approach in preparation of double negative cells. These authors implemented a density gradient centrifugation as an initial step in purifying progenitor thymocytes thus potentially depleting certain populations of interest.

A prerequisite for successful T–cell development is availability of a specialised microenvironment. This is provided by the thymus. Thymic epithelia provide a plethora of signals at various stages of thymocyte development ranging from secretion of soluble factors to direct cell–cell interactions. In turn, development of the thymic stroma is heavily reliant on the presence of developing thymocytes. In mice where T–cell development is blocked at the earliest stage, DN1, by an introduction of a human CD3ε transgene (Wang, Biron et al. 1994), there is a severely compromised thymic architecture (Hollander, Wang et al. 1995) with no clear distinction between the cortex and the medulla. It seems that the formation of a functional cortex is dependant on DN2/DN3 cells, since analysis of the *Rag-2* null thymus, where T–cell development is blocked at the DN3 stage, revealed a densely packed and normally organised cortex, but an underdeveloped medulla (Hollander, Wang et al. 1995). Complete restoration of the medulla is achieved by introducing cells, which are able to differentiate past the DN3

stage to yield thymocytes expressing a functional TCR (van Ewijk, Hollander et al. 2000; van Ewijk, Shores et al. 1994).

The imbalance in the dynamics of progenitor pools observed in mutant mice that were analysed in this study might be a consequence of the disturbed thymic environment. A grave decrease in cellularity and an altered phenotype of the earliest T–cell progenitors in mutants suggests that the signals delivered by the thymic stroma are abnormal. In *IL-7* null mutants the likely candidate is IL-7 itself, thus further underlining the importance of this cytokine in T–cell development. In genetic models where mutations are intrinsic to the T–cell lineage, the involvement of the epithelial compartment is highlighted. The DN1 compartment of *Rag-2* deficient mice lacks a notable population of CD117+ and a near absence of CD45R+ cells, which suggests that these populations are strongly dependant on signals emanating from the medulla. Since T–cell progenitors enter the thymus at the cortico-medullary junction, the absence of a given signal results in a lack of proliferation and generation of the respective progenitor population. Interestingly, the $CD127^{high}CD90.2^{high}$ population seems to be unaffected, which implies that this population requires an alternative set of signals, which govern its sustenance. On the other hand, the disruption in the earliest progenitor pools might be a consequence of a complete lack of more mature T–cells, which could also supply survival signals to their immature counterparts.

Divergence in genetic background of different mouse strains results in a variation of a functional response. This includes susceptibility to infectious agents, such as Toxoplasma gondii (Lee, Kasper, 2004), development of coronary disease (Tabibiazar, Wagner et al. 2005) and ability to regenerate tissue (Shi, Wakil et al. 1997). Differences in frequency and function of plasmacytoid dendritic cells (Asselin-Paturel, Brizard et al. 2003), HSCs (Morrison, Qian et al. 2002) and progenitor B– and T–cells (Shortman,

Wilson 1988; Rolink, Grawunder et al. 1994) have been illustrated between mouse strains. Analysis of T–cell progenitor distribution between four commonly used laboratory mouse strains revealed that there are considerable differences, both in frequency as well as number of double negative thymocytes. The C57BL/6 mouse strain is the most widely used in studying T–cell development, however, this strain exhibits a composition of progenitor subsets, which is not comparable to CBA, BALB/c and C3He mouse strains. The DN1 CD117 population predominates the DN1 pool of C57BL/6, whereas in all other strains analysed the CD45R population was the preponderant fraction. These differences could arise due to different cell cycle properties between progenitors of different mouse strains as a result of differential requirements for and responses to cytokines and growth factors, which in turn, have a varying pattern of expression between different mouse strains (Charles, Weber et al. 1999). The preponderance of the DN1 CD45R population in 3 out of the 4 mouse strains analysed may suggest that these cells have a relevant function during T-cell development, for example as an alternative intermediate.

Chapter 4

Lineage Tracing of Progenitor Thymocytes

Introduction

The relationship between early thymic progenitors and progenitor populations in the bone marrow has not been directly established. To understand the relationship between double negative thymocytes and bone marrow progenitor cells capable of generating T-lineage progeny, a fluorescent reporter system was implemented (Figure 4.1). In this system, the fluorescent reporter was only active once the stop signal preceding the fluorescent protein coding sequence was removed by the action of *Cre* recombinase, which was driven by the human CD2 promoter and locus control regions (hCD2::iCre). Upon excision of the stop sequence, the enhanced yellow fluorescent protein (EYFP) is expressed, permanently marking all cells and their progeny, thus potentially identifying intermediate developmental stages. Previous studies have demonstrated that this reporter system is active only in the lymphoid lineage of the haematopoietic system (de Boer, Williams et al. 2003).

Fluorescent reporter expression during haematopoietic development has so far been successful in identifying the early lymphoid progenitor (ELP) population (Igarashi, Gregory et al. 2002). In that system, the fluorescent protein was reporting the activity of the endogenous *Rag-1* locus. That system would identify cells where *Rag-1* was expressed at a particular moment in time, therefore ondulation in *Rag-1* gene expression may not have revealed the subsequent stages of ELP differentiation.

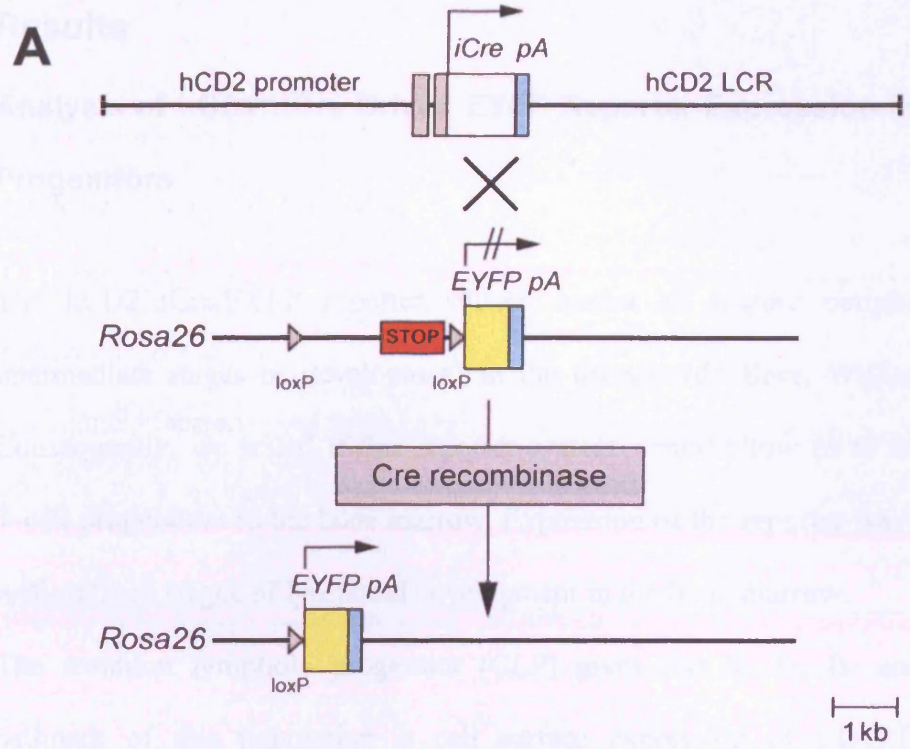
A fluorescent reporter system, which permanently marked all cells and their progeny has also been used to understand haematopoietic development (Ye, Iwasaki et al. 2003). This study demonstrated that expression of lysozyme M, a myeloid affiliated gene, in long-term repopulating stem cells, did not necessarily commit these cells to the respective lineage. Instead, promiscuous expression of lineage affiliated genes in multipotent progenitors preceded final commitment.

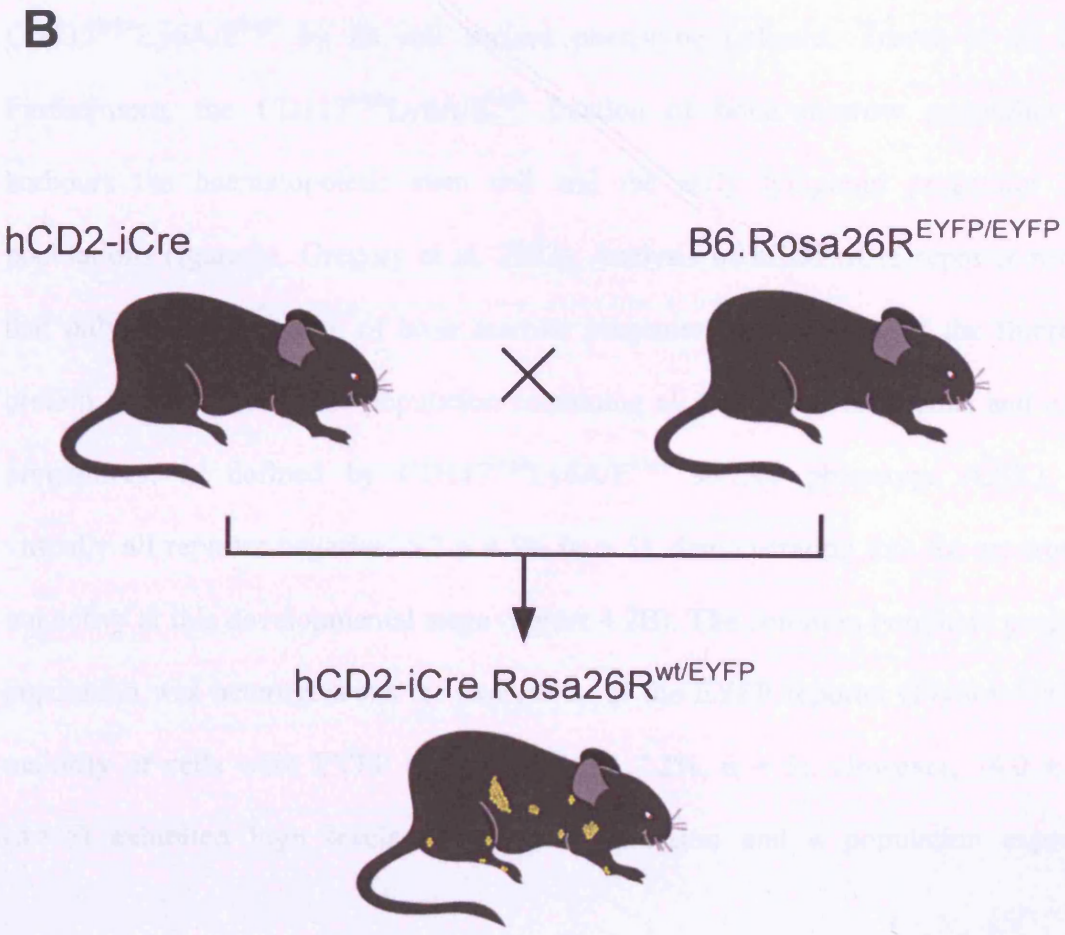
Since EYFP expression driven by the human CD2 improved Cre has so far been shown to be expressed exclusively in the lymphoid lineage, the aim was to test whether this reporter system could mark intermediate stages in lymphoid development and therefore aid in establishing a relationship between thymic and bone marrow progenitor cells. Additionally, relationship between various thymic progenitor populations was addressed.

Figure 4.1 Human CD2::iCre EYFP Reporter

A Expression of the *Cre* recombinase is driven by the human CD2 promoter and locus control regions. This transgenic line (hCD2::iCre) was generated by de Boer and Williams *et al.* (de Boer, Williams et al. 2003). The reporter strain (R26REYFP) was generated by insertion of the enhanced yellow fluorescent protein (EYFP) cDNA, preceded by a loxP-flanked transcriptional stop sequence, into the ROSA26 locus. This strain was generated by Srinivas *et al.* (Srinivas, Watanabe et al. 2001). Upon expression of *Cre* recombinase, the loxP-flanked stop sequence is removed therefore permitting expression of EYFP, thus permanently marking all cells and their progeny.

B Generation of the hCD2::iCre EYFP reporter strain. The hCD2::iCre transgenic line was crossed to homozygous R26REYFP strain, thereby generating hCD2::iCre.EYFP reporter strain, where all cells that have expressed the *Cre* recombinase are permanently marked by EYFP. In addition, all progeny of EYFP+ cells are also permanently marked.


A
iCre pA
hCD2 promoter
hCD2 LCR
EYFP pA
Rosa26
STOP
loxP
loxP
Cre recombinase
EYFP pA
Rosa26
loxP
1 kb


B
hCD2-iCre
B6.Rosa26R EYFP/EYFP
hCD2-iCre Rosa26R wt/EYFP

Results

Analysis of hCD2::iCre Driven EYFP Reporter Expression in Bone Marrow Progenitors

The hCD2::iCre.EYFP reporter system marks all mature peripheral T-cells and intermediate stages of development in the thymus (de Boer, Williams et al., 2003). Consequently, we tested if this reporter system would allow us to trace the origin of T-cell progenitors to the bone marrow. Expression of the reporter was first examined in well-defined stages of lymphoid development in the bone marrow.

The common lymphoid progenitor (CLP) gives rise to T-, B- and NK-cells. The hallmark of this population is cell surface expression of CD127, in addition to intermediate levels of CD117 and Ly6A/E (Kondo Weissman et al. 1997). The CLP is thought to derive from the multipotent progenitor population, which is defined as $CD117^{high}Ly6A/E^{high}$ by its cell surface phenotype (Akashi, Traver et al. 2000). Furthermore, the $CD117^{high}Ly6A/E^{high}$ fraction of bone marrow progenitor cells harbours the haematopoietic stem cell and the early lymphoid progenitor (ELP) populations (Igarashi, Gregory et al. 2002). Analysis of hCD2::iCre reporter revealed that only a small fraction of bone marrow progenitor cells expressed the fluorescent protein (Figure 4.2A). The population containing all functional stem cells and earliest progenitors, as defined by $CD117^{high}Ly6A/E^{high}$ surface phenotype (LSK), were virtually all reporter negative, 5.2 ± 4.5% (n = 5), demonstrating that the reporter was not active at this developmental stage (Figure 4.2B). The common lymphoid progenitor population was heterogeneous for expression of the EYFP reporter (Figure 4.2C), the majority of cells were EYFP negative (77.0 ± 7.2%, n = 5). However, 14.0 ± 6.2% (n = 5) exhibited high levels of reporter expression and a population expressing

intermediate levels was revealed, (7.9 ± 3.5%, n = 5), suggesting that recent recombination of the *Rosa26* locus took place. In addition this illustrated that the common lymphoid progenitor population is heterogeneous. Interestingly, the largest population of EYFP+ bone marrow progenitors resided in the $CD117^{intermediate}Ly6A/E^{high}$ fraction and did not express CD127 (Figure 4.2D). This population could be a downstream progeny of the EYFP+ CLP fraction or a separate lymphoid progenitor pool.

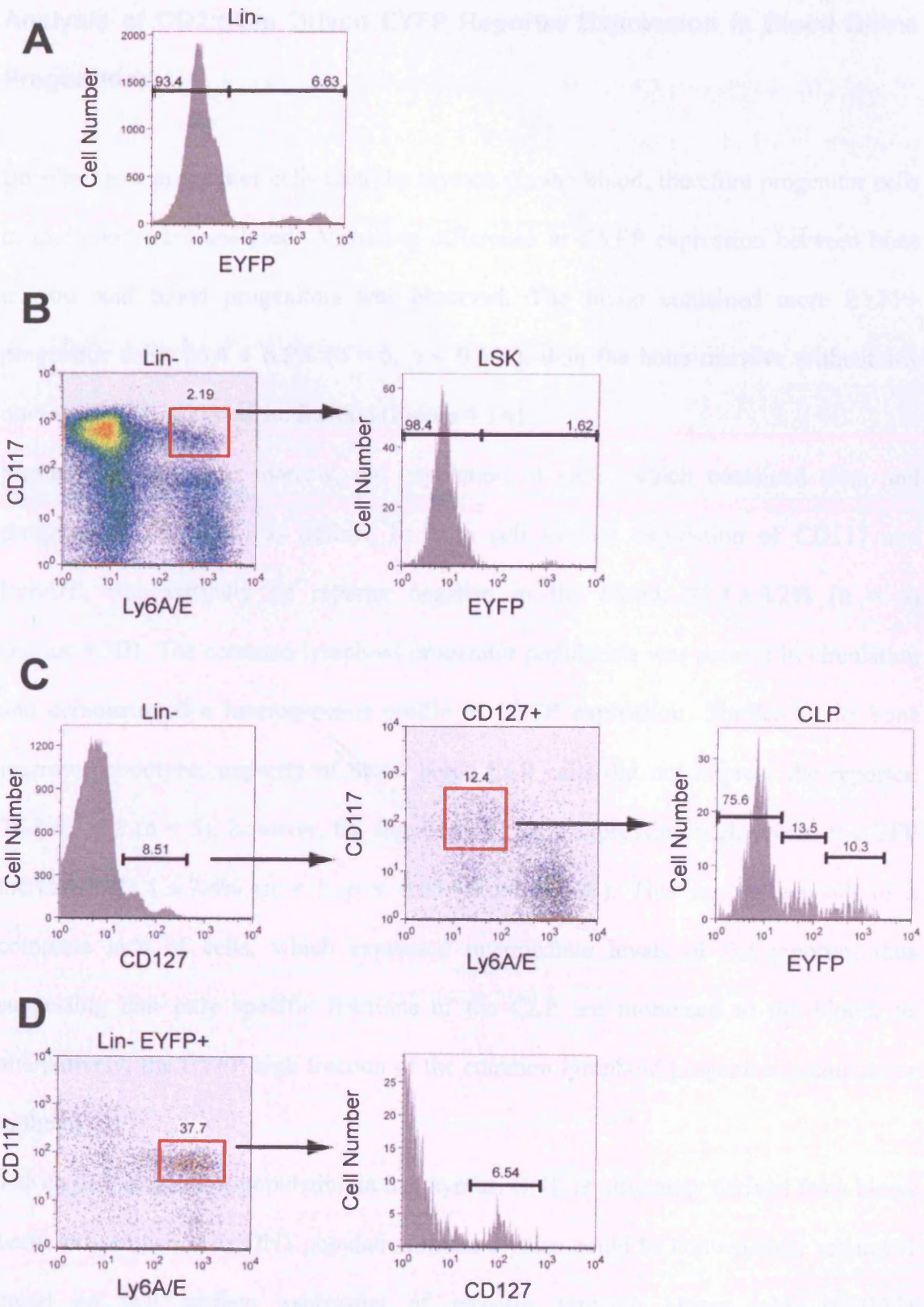
Figure 4.2 Analysis of hCD2::iCre Driven EYFP Reporter Expression in Bone Marrow Progenitor Cells.

A A representative histogram from five independent experiments illustrating expression of hCD2::iCre driven reporter in lineage negative (Lin-) bone marrow progenitor cells from 4-8 weeks old hCD2::iCre.EYFP mice. Total bone marrow was labelled with a cocktail of biotinylated lineage antibodies, which included CD8α, CD3ε, CD45R, NK1.1, CD11b, Ter119 and Ly6G, and were subsequently removed by magnetic depletion. Only a small population of progenitor cells expressed the reporter in the bone marrow.

B A representative histogram illustrating the composition of Lin- bone marrow progenitor cells, as defined by cell surface expression of CD117 and Ly6A/E. Haematopoietic stem cells and multipotent progenitors were defined as $CD117^{high}Ly6A/E^{high}$ and illustrated a near absence of reporter expression.

C A gating sequence illustrating expression of EYFP reporter in the common lymphoid progenitor (CLP) population. The CLP was defined as $Lin\text{-}CD127+CD117^{intermediate}Ly6A/E^{intermediate}$. The CLP was heterogeneous in the expression of the EYFP reporter, majority of cells were negative. However intermediate and high levels of EYFP were also detected in this population.

D A representative histogram illustrating the composition of Lin-EYFP+ bone marrow progenitor cells as defined by cell surface expression of CD117 and Ly6A/E. The largest population had intermediate levels of CD117, high levels of Ly6A/E and by and large did not express CD127.


A
Lin-
Cell Number
93.4
6.63
EYFP
B
Lin-
CD117
2.19
Ly6A/E
LSK
Cell Number
98.4
1.62
EYFP
C
Lin-
Cell Number
8.51
CD127
CD127+
CD117
12.4
Ly6A/E
CLP
Cell Number
75.6
13.5
10.3
EYFP
D
Lin- EYFP+
CD117
37.7
Ly6A/E
Cell Number
6.54
CD127

Analysis of CD2::iCre Driven EYFP Reporter Expression in Blood Borne Progenitors

Bone marrow progenitor cells seed the thymus via the blood, therefore progenitor cells in the blood were analysed. A striking difference in EYFP expression between bone marrow and blood progenitors was observed. The blood contained more EYFP+ progenitor cells, 56.4 ± 6.5% (n = 5, $p < 0.001$), than the bone marrow, without any obvious EYFP intermediate fraction (Figure 4.3A).

Similarly to the bone marrow, the population of cells, which contained stem and progenitor cells (LSK) as defined by high cell surface expression of CD117 and Ly6A/E, was virtually all reporter negative in the blood, 95.4 ± 3.2% (n = 5) (Figure 4.3B). The common lymphoid progenitor population was present in circulation and demonstrated a heterogeneous profile of EYFP expression. Similar to the bone marrow phenotype, majority of blood borne CLP cells did not express the reporter, 78.8 ± 7.4% (n = 5), however, the frequency of cells expressing high levels of EYFP increased, 21.1 ± 7.4% (n = 5, $p < 0.05$) (Figure 4.3B). This was as a result of a complete lack of cells, which expressed intermediate levels of the reporter, thus suggesting that only specific fractions of the CLP are mobilised to the blood, or alternatively, the EYFP high fraction of the common lymphoid progenitor accumulates in the blood.

The earliest progenitor population in the thymus, DN1, is ultimately derived from blood borne progenitors. The DN1 population in the thymus could be conveniently separated based on cell surface expression of receptor tyrosine kinase c-kit (CD117) (see Chapter 3). Consequently, expression of the fluorescent reporter was correlated with expression of CD117 in blood borne progenitors. The EYFP expression was completely distinct from expression of CD117 (Figure 4.3C). The CD117+ cells in the

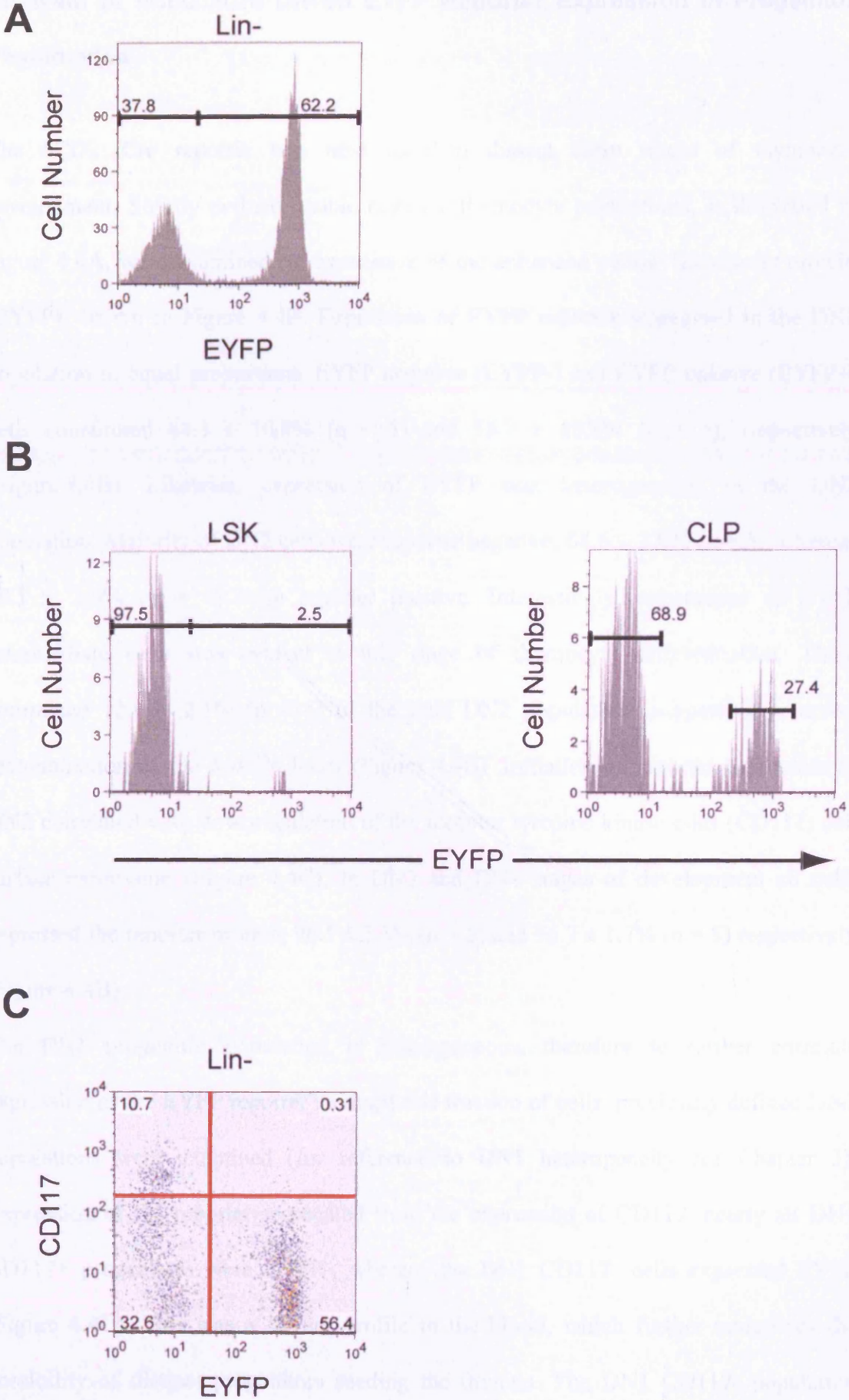
blood were completely reporter negative, whereas EYFP+ cells did not express CD117 (Figure 4.3C). This demonstrates that blood borne progenitors are heterogeneous and comprise distinct populations as assessed by CD117 and EYFP expression. This suggests that heterogeneity observed in the DN1 population (see Chapter 3) could be a direct result of distinct progenitor populations colonising the thymus.

Figure 4.3 Analysis of hCD2::iCre Driven EYFP Reporter Expression in Blood Borne Progenitors.

A A representative histogram from five independent experiments illustrating expression of the hCD2::iCre driven reporter in lineage negative (Lin-) blood progenitor cells from 4-8 weeks old hCD2::iCre.EYFP mice. Total blood was labelled with a cocktail of biotinylated lineage antibodies, which included CD8α, CD3ε, CD45R, NK1.1, CD11b, Ter119 and Ly6G, and labelled cells were subsequently removed by magnetic depletion. Expression of the fluorescent reporter is heterogeneous in blood progenitors with majority of cells expressing the reporter.

B Representative histograms illustrating expression of the reporter protein in defined progenitor populations. Haematopoietic stem cells and multipotent progenitors (LSK) are defined as $CD117^{high}Ly6A/E^{high}$ and do not express EYFP. The common lymphoid progenitor (CLP), defined as CD127+ $CD117^{intermediate}Ly6A/E^{intermediate}$, is heterogeneous for EYFP expression, majority of cells do not express the fluorescent protein.

C Representative histograms illustrating the composition of lineage negative circulating progenitors as assessed by CD117 and EYFP expression. Expression of CD117 segregates from expression of EYFP, EYFP positive cells do not express CD117, whereas high expressors of CD117 do not express EYFP.


A
Lin-
Cell Number
37.8
62.2
EYFP
B
LSK
CLP
Cell Number
97.5
2.5
68.9
27.4
EYFP
C
Lin-
CD117
10.7
0.31
32.6
56.4
EYFP

Analysis of hCD2::iCre Driven EYFP Reporter Expression in Progenitor Thymocytes

The hCD2::iCre reporter was next used to dissect early stages of thymocyte development. Strictly defined double negative thymocyte populations, as illustrated in Figure 4.4A, were examined for expression of the enhanced yellow fluorescent protein (EYFP), shown in Figure 4.4B. Expression of EYFP reporter segregated in the DN1 population in equal proportions. EYFP negative (EYFP-) and EYFP positive (EYFP+) cells constituted 44.3 ± 10.8% (n = 5) and 55.7 ± 10.8% (n = 5), respectively (Figure 4.4B). Likewise, expression of EYFP was heterogeneous in the DN2 population. Majority of DN2 cells were reporter negative, 68.6 ± 2.0% (n = 5), whereas 19.1 ± 2.6% (n = 5) were reporter positive. Interestingly, appearance of EYFP intermediate cells was evident at this stage of thymocyte differentiation. These comprised 12.6 ± 2.1% (n = 5) of the total DN2 population, suggesting in active recombination of the *Rosa26* locus (Figure 4.4B). Initiation of reporter expression in DN2 correlated with downregulation of the receptor tyrosine kinase c-kit (CD117) cell surface expression (Figure 4.4C). In DN3 and DN4 stages of development all cells expressed the reporter protein, 96.1 ± 2.5% (n = 5) and 96.7 ± 1.7% (n = 5) respectively (Figure 4.4B).

The DN1 progenitor population is heterogeneous, therefore to further correlate expression of the EYFP reporter amongst this fraction of cells, previously defined DN1 populations were examined (for reference to DN1 heterogeneity see Chapter 3). Expression of the reporter segregated from the expression of CD117, nearly all DN1 CD117+ progenitors were EYFP-, whereas the DN1 CD117- cells expressed EYFP (Figure 4.4C). This was a similar profile to the blood, which further underlines the possibility of distinct progenitors seeding the thymus. The DN1 CD117- population

contains a preponderant fraction of cells, which express CD45R and a small fraction, which express CD127 (see Chapter 3). The DN1 CD45R+ population was uniformly EYFP positive, whereas the DN1 CD127+ fraction, which by its cell surface phenotype resembles the common lymphoid progenitor, comprised a majority of cells, which did not express the reporter and furthermore, no evidence of EYFP intermediate cells was apparent (Figure 4.4D), which was a similar profile seen in the blood.

Since expression of the hCD2::iCre reporter system has so far been reported in the lymphoid lineage only (de Boer, Williams et al., 2003), homogeneous expression of EYFP amongst the DN1 CD45R population would suggest commitment of this population to the lymphoid or the T-cell lineage. Noteworthy, is the presence of a small, yet detectable, fraction of EYFP+ cells amongst the DN1 CD117+ population, (Figure 4.4C), which illustrates the heterogeneity of this population and implies the existence of lymphoid or T-cell lineage committed cells amongst this compartment of progenitor thymocytes.

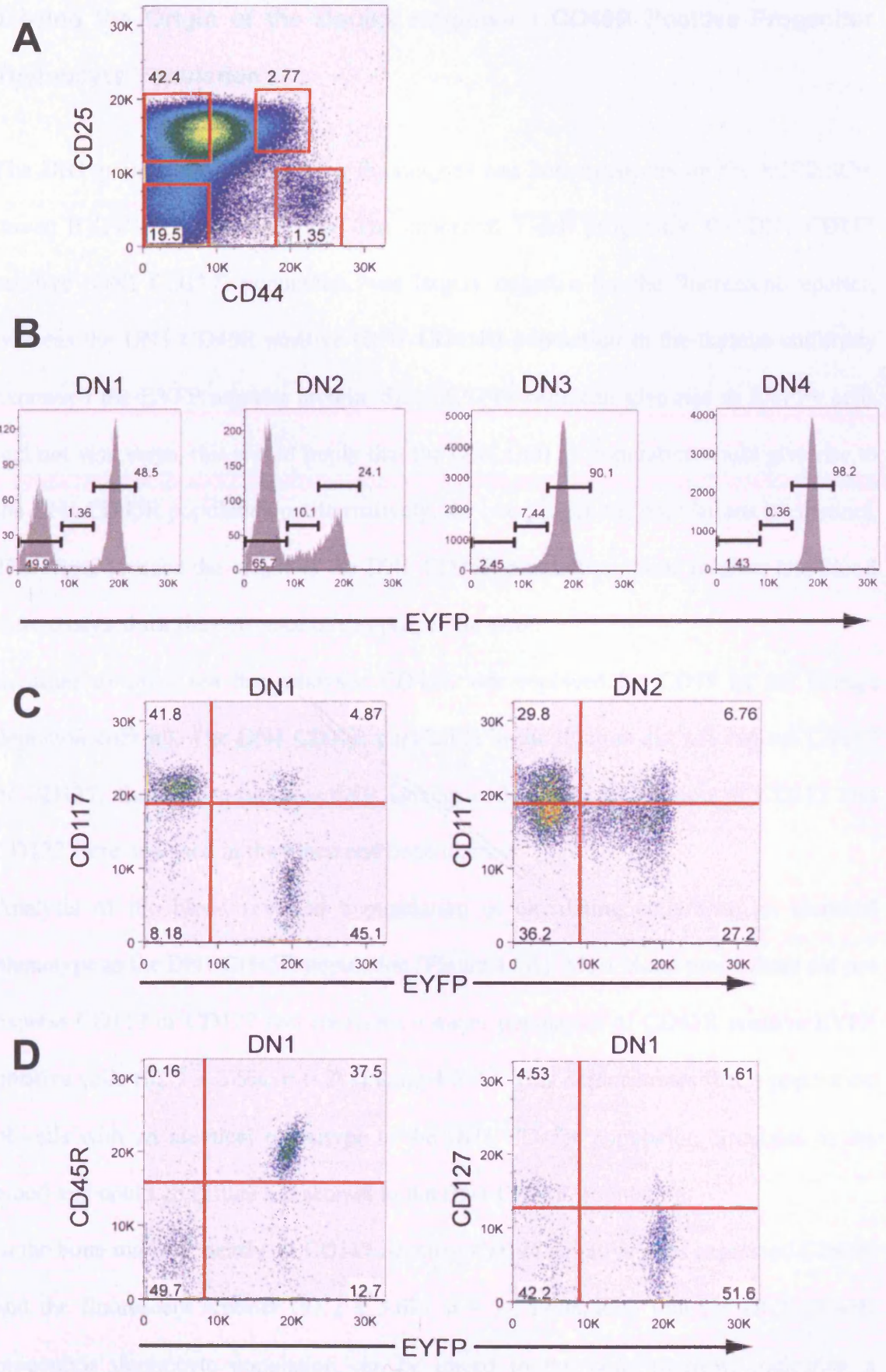
Figure 4.4 Analysis of hCD2::iCre Driven EYFP Reporter Expression in Double Negative Thymocytes.

A A representative histogram from five independent experiments illustrating expression of CD44 and CD25 in DN thymocytes from 4-8 weeks old hCD2::iCre.EYFP mice. Strictly positioned gates were used to define double negative progenitor thymocyte populations. These definitions were subsequently used to examine expression of the hCD2::iCre driven EYFP reporter amongst double negative thymocytes.

B Expression of hCD2 iCre reporter in double negative thymocytes as defined in **A**. Expression of the reporter segregates in DN1 and DN2 populations, whereas all cells express the reporter at DN3 and DN4 stages of development.

C Expression of fluorescent reporter in DN1 and DN2 populations as resolved by CD117 expression. Majority of CD117+ cells do not express the reporter, whereas CD117- cells are mostly reporter positive. DN2 cells are heterogeneous with an obvious fraction expressing intermediate levels of the fluorescent protein.

D Expression of hCD2::iCre driven reporter amongst previously defined DN1 populations. All DN1 CD45R+ progenitors express EYFP, whereas cells expressing CD127 show heterogeneous profile of reporter expression, majority are negative.


A
CD25
CD44
42.4
2.77
19.5
1.35
B
DN1
DN2
DN3
DN4
48.5
1.36
24.1
10.1
90.1
7.44
98.2
0.36
EYFP
C
DN1
DN2
CD117
41.8
4.87
8.18
45.1
29.8
6.76
36.2
27.2
EYFP
D
DN1
DN1
CD45R
CD127
0.16
37.5
49.7
12.7
4.53
1.61
42.2
51.6
EYFP

Tracing the Origin of the Double Negative 1 CD45R Positive Progenitor Thymocyte Population

The DN1 population of progenitor thymocytes was heterogeneous for the hCD2::iCre driven EYFP reporter expression. The canonical T-cell progenitor, the DN1 CD117 positive (DN1 CD117) population, was largely negative for the fluorescent reporter, whereas the DN1 CD45R positive (DN1 CD45R) population in the thymus uniformly expressed the EYFP reporter protein. Since EYFP- cells can give rise to EYFP+ cells and not visa versa, this would imply that the DN1 CD117 population could give rise to the DN1 CD45R population or alternatively, the two progenitor populations are distinct. Therefore, to trace the origin of the DN1 CD45R population, bone marrow and blood were analysed for the existence of this progenitor pool.

In order to carry out this analysis, CD45R was replaced by CD19 in the lineage depletion cocktail. The DN1 CD45R population in the thymus did not express CD117 or CD127, therefore progenitor cells lacking cell surface expression of CD117 and CD127 were analysed in the blood and bone marrow.

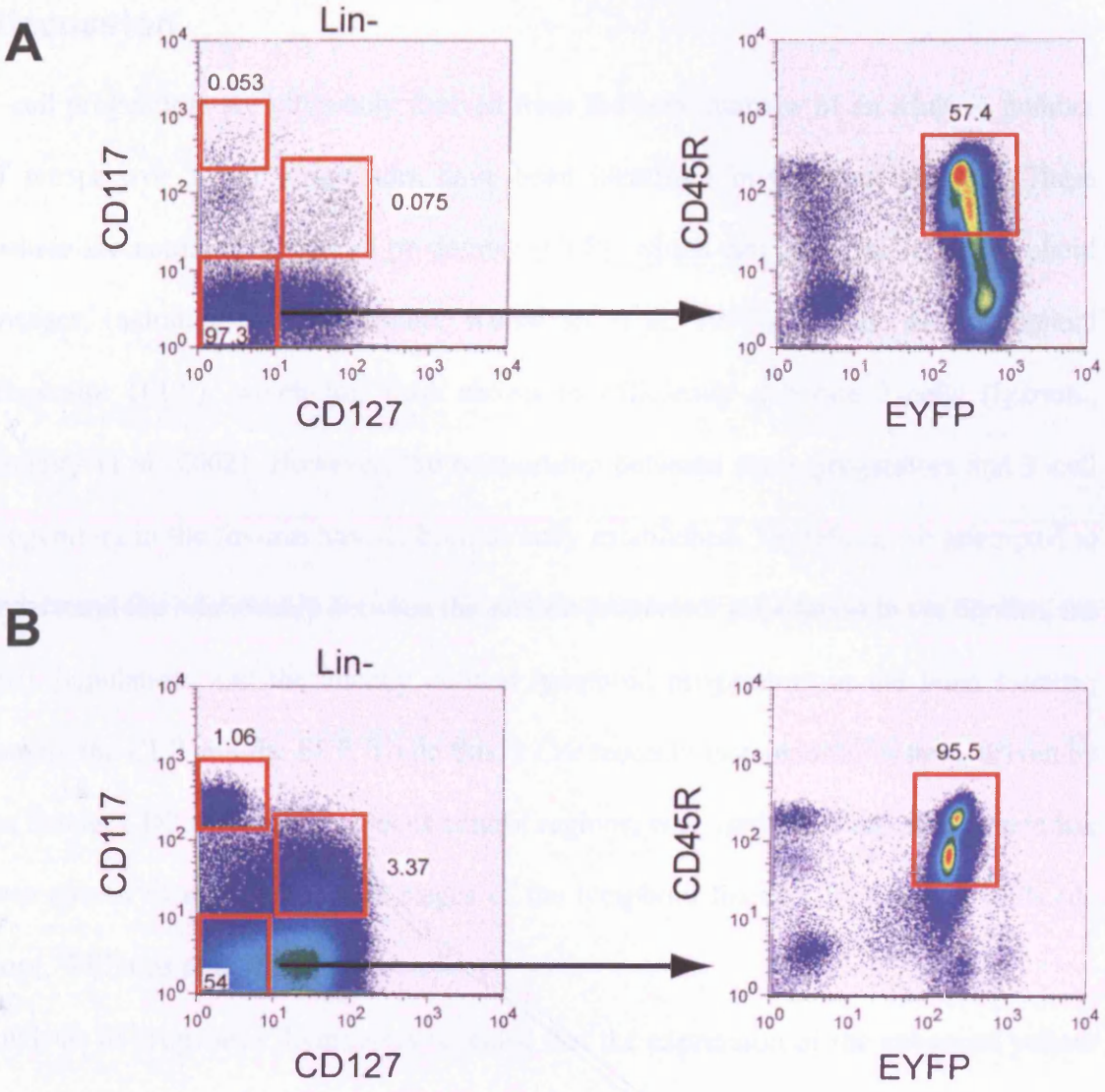
Analysis of the blood revealed a population of circulating cells with an identical phenotype to the DN1 CD45R population (Figure 4.5A). Most blood progenitors did not express CD117 or CD127 and contained a major population of CD45R positive EYFP positive cells (62.9 ± 5.5%, n = 2) (Figure 4.5 A). This demonstrates that a population of cells with an identical phenotype to the DN1 CD45R population circulates in the blood and could constitute a precursor to the DN1 CD45R population.

In the bone marrow, nearly all CD117 negative CD127 negative cells expressed CD45R and the fluorescent reporter (91.2 ± 3.6%, n = 2), illustrating that the DN1 CD45R progenitor thymocyte population can be traced to the bone marrow, indicating a different origin to the DN1 CD117 population (Figure 4.5C).

Figure 4.5 Lineage Tracing of DN1 CD45R Population in Blood and Bone Marrow.

A A representative histogram from two independent experiments illustrating the composition of lineage negative blood borne progenitors resolved by cell surface expression of CD117 and CD127 from 4-8 weeks old hCD2::iCre.EYFP mice. Total blood was labelled with a cocktail of biotinylated lineage antibodies, which included CD8α, CD3ε, CD19, NK1.1, CD11b, Ter119 and Ly6G, and labelled cells were subsequently removed by magnetic depletion. Analysis of CD117 negative CD127 negative progenitors reveals a large population expressing CD45R and EYFP.

B A representative histogram from two independent experiments illustrating the composition of lineage negative bone marrow progenitors resolved by cell surface expression of CD117 and CD127 from 4-8 weeks old hCD2::iCre.EYFP mice. Total bone marrow was labelled with a cocktail of biotinylated lineage antibodies, which included CD8α, CD3ε, CD19, NK1.1, CD11b, Ter119 and Ly6G, and labelled cells were subsequently removed by magnetic depletion. Analysis of CD117 negative CD127 negative progenitors reveals that nearly all cells express CD45R and EYFP.


A
Lin-
0.053
0.075
97.3
CD117
CD127
57.4
CD45R
EYFP
B
Lin-
1.06
3.37
54
CD117
CD127
95.5
CD45R
EYFP

Discussion

T-cell progenitors are ultimately derived from the bone marrow of an adult. A number of prospective T-cell progenitors have been identified in the bone marrow. These include the common lymphoid progenitor (CLP), which can give rise to all lymphoid lineages, including T-cells (Kondo, Weissman et al. 1997), and the early lymphoid progenitor (ELP), which has been shown to efficiently generate T-cells (Igarashi, Gregory et al. 2002). However, the relationship between these progenitors and T-cell progenitors in the thymus has not been directly established. Therefore, we attempted to understand the relationship between the earliest progenitor population in the thymus, the DN1 population, and the already defined lymphoid progenitors in the bone marrow, namely the CLP and the ELP. To do this, a *Cre* recombinase reporter system, driven by the human CD2 promoter and locus control regions, was used. This reporter system has been shown to mark all mature stages of the lymphoid lineage, including T-cells (de Boer, Williams et al., 2003).

Analysis of progenitor thymocytes revealed that the expression of the enhanced yellow fluorescent protein (EYFP) reporter correlated well with the irreversible commitment to the T-cell lineage. At the double negative 3 (DN3) stage, all cells were marked by EYFP expression, which identifies this reporter system as a useful tool to indicate the status of commitment to the T-cell lineage.

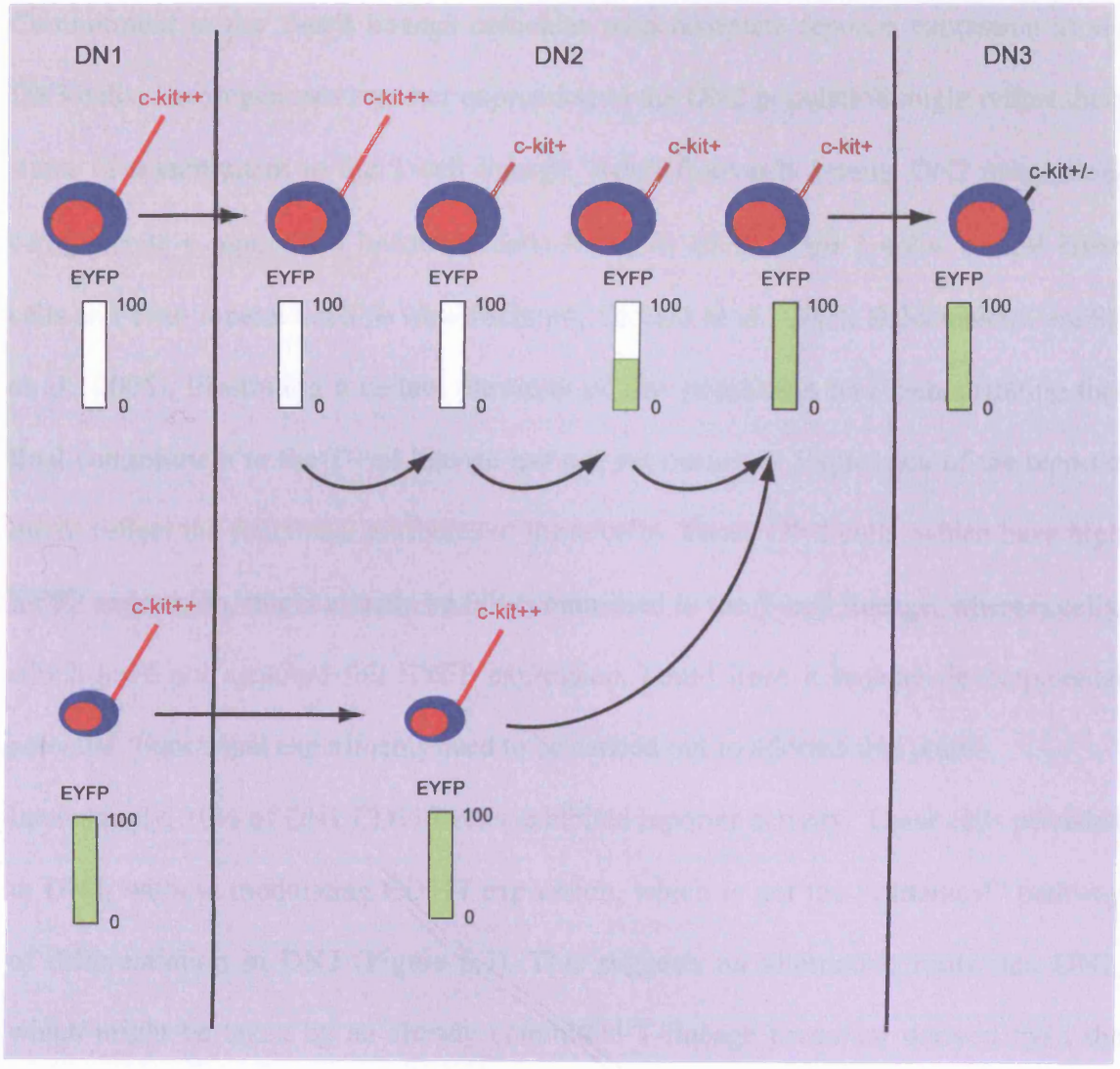
T-cell development in the thymus begins at the DN1 stage, furthermore, it is currently believed that the most robust T-cell differentiation potential resides in the fraction that exhibits cell surface expression of the receptor tyrosine kinase c-kit (CD117). Thus the DN1 CD117 positive (DN1 CD117) population ultimately gives rise to DN2 and, subsequently, DN3 cells. Analysis of reporter expression amongst DN1 CD117 cells revealed that, on the whole, these cells were not marked by the fluorescent reporter. The

DN2 population was heterogeneous in terms of reporter expression, majority of cells were negative, with a smaller fraction expressing high reporter levels. However, a fraction of cells was detected, which exhibited intermediate levels of reporter expression, suggesting a recent recombination event. This correlated with a slight downregulation of CD117, and a further upregulation of EYFP. Since only EYFP- cells can give rise to EYFP+ cells, and not vice versa, a sequence of additional differentiation events could be inferred from the analysis of fluorescent reporter expression during the early stages of T-cell differentiation. This is illustrated in Figure 6.5. DN1 CD117 cells, which are reporter negative, upregulate CD25 and thus progress to the DN2 stage. At this point EYFP expression is not detected. Upon downregulation of CD117, cells begin to express EYFP, which is consequently further upregulated. Downmodulation of CD44 and CD117 accompanies the cells to the DN3 stage, where all are reporter positive.

Figure 4.6 A Model of Canonical T-cell Developmental Progression.

A model of T-cell development in the thymus based on analysis of CD2 iCre reporter expression in DN1 CD117, DN2 and DN3 populations. The main developmental progression of progenitor thymocytes takes place from DN1 CD117 cells, which are completely reporter positive. Upregulation of CD25 accompanies the cells to the DN2 stage, where the earliest DN2 cells express high levels of c-kit (CD117) and no reporter. The next DN2 intermediate is marked by downregulation of CD117, where the cells still do not express the fluorescent reporter. Subsequently, cells begin to express low levels of fluorescent protein, which is further upregulated marking late DN2 stage. Downregulation of CD44 and CD117 defines the DN3 stage, where all cells express the reporter.

An additional population in the DN1 CD117 fraction exhibited reporter expression. This population persisted in DN2 without downmodulation of CD117. If this population is relevant in T-cell development, then these cells automatically bypass the early and intermediate stages of DN2 and join the conventional pathway of development at the late DN2 stage.


DN1
DN2
DN3
c-kit++
c-kit++
c-kit+
c-kit+
c-kit+
c-kit+/-
EYFP
100
0
c-kit++
c-kit++

Commitment to the T-cell lineage coincides with complete reporter expression in all DN3 cells. Heterogeneous reporter expression in the DN2 population might reflect their status of commitment to the T-cell lineage. Apart from α/β T-cells, DN2 progenitors can generate a number of haematopoietic lineages, such as γ/δ T-cells, natural killer cells and even myeloid cell *in vitro* (Schmitt, Ciofani et al., 2004; Balciunaite, Ceredig et al., 2005), illustrating a certain plasticity of this population and demonstrating that final commitment to the T-cell lineage has not yet occurred. Expression of the reporter might reflect the functional attributes of these cells. Those DN2 cells, which have high EYFP expression, might already be fully committed to the T-cell lineage, whereas cells, which have not acquired full EYFP expression, could have a broader developmental potential. Functional experiments need to be carried out to address this point.

Interestingly, 10% of DN1 CD117 cells exhibited reporter activity. These cells persisted in DN2, without modulating CD117 expression, which is not the "canonical" pathway of differentiation in DN2 (Figure 6.5). This suggests an alternative route into DN2, which might be taken by an already committed T-lineage precursor derived from the DN1 CD117 fraction.

T-cell progenitors must seed the thymus via the blood. Analysis of fluorescent reporter expression in the blood revealed that distinct progenitor populations are in circulation. Cells expressing high levels of CD117 were detected, which were completely devoid of any reporter expression. This correlates well with the phenotype of the DN1 CD117 population in the thymus and therefore points to a precursor product relationship between these cells. However, in contrast to some published reports (Schwarz, Bhandoola 2004), the common lymphoid progenitor (CLP) was found in circulation. Similarly to the DN1 CD117 population, most CLP cells have not yet activated the fluorescent marker in the blood or the bone marrow, therefore to rule out the

contribution of the CLP to the DN1 CD117 population, based on reporter expression, is gratuitous. The general phenotype of the CLP, in addition to a "CLP-like" population found in the thymus would contest a direct relationship between the DN1 CD117 and the CLP population in the blood and bone marrow. The role of the "CLP-like" population in the thymus during thymic ontogenesis would need to be resolved. The "CLP-like" population might constitute an intermediate stage between the DN1 CD117 population and the CLP found in blood or bone marrow. The most probable precursor to the thymic DN1 CD117 population, however, would be CD117+EYFP- cells in the blood and the respective population in the bone marrow.

The population of cells, which does not express CD117 in the DN1 fraction, has largely been ignored due to its inability to generate progeny *in* vivo (Allman, Sambandam et al. 2003), even though capacity to generate T-cells has been detected *in vitro* (Porritt, Rumfelt et al. 2004). Remarkably, nearly all DN1 CD117- cells exhibited high levels of hCD2::iCre driven reporter expression, without an obvious intermediate fraction, suggesting a distant recombination event. As discussed in the previous chapter, methods to isolate double negative progenitors vary, thus distorting the composition of early subsets. Our phenotypic study revealed a population of cells in the DN1 compartment which did not express CD117 but harboured cell surface expression of CD45R (DN1 CD45R, see Chapter 3). Analysis of fluorescent reporter expression amongst this fraction identified these cells as ubiquitously positive. This level of reporter expression would align the DN1 CD45R population with late DN2 or DN3 stages of T-cell differentiation and suggest that the adult thymus can be seeded by already lineage committed progenitor cells. The origin of the DN1 CD45R population could be traced to the bone marrow, therefore implying that commitment to the T-cell lineage can occur prethymically.

Chapter 5

Gene Expression Analysis of Progenitor Thymocytes

Introduction

The functional capacity of any given cell in a multicellular organism is ultimately determined by the genetic programs active within that cell. Although posttranscriptional mechanisms are important in regulating the expression of many genes, most cellular regulation is achieved by changes in mRNA levels. Natural selection has acted to optimise simultaneously the functional properties of the product encoded by the gene and the program that dictates the spatial, temporal and quantitative aspects of gene activity. In general, each gene is expressed in the specific cells and under specific conditions in which its product makes a contribution to fitness. Therefore, even subtle variations in the expression patterns of genes can be related to corresponding differences in the functions of the products they encode.

Approaches which utilise genome wide screening have now been routinely used to try and understand molecular events taking place during disease states, effects of specific drugs or stimuli and effects of specific mutations. In addition, global gene expression profiles have been implemented to establish a complete transcriptome on the basis of which a specific cellular population can be identified (Ivanova, Dimos et al. 2002). In the haematopoietic system, comparative studies have been put into practice to elucidate the molecular events taking place during the differentiation process of a HSC towards a more committed blood cell lineage (Terskikh, Miyamoto, et al. 2003). These studies have illustrated that specific and distinct gene clusters are active during the formation of divergent progenitor cell. Furthermore, the genetic programs active in various progenitor cells mirror their functional capacity. In other words, myeloid progenitors express genes exclusively related to the myeloid lineage, whereas lymphoid progenitors show activity of those genes which are functionally related only to the lymphoid lineage, thus genetically separating the two branches of the haematopoietic system

(Akashi, He et al. 2003). Microarray studies focusing on T-cell development in the thymus have been reported. One such study demonstrated a limited number of regulated genes during the developmental process and did not provide any information on the expression pattern of genes within the earliest T-lineage progenitor, the DN1 CD117^{+} population (ETP) (Hoffmann, Bruno et al 2003). Another study solely focused on the regulation of transcription factors throughout T-cell development, however, the phenotype of the earliest T-cell progenitor was inconsistent with the accepted definition (Tabrizifarad, Olaru et al. 2004).

In this study, the ability to relate a gene expression signature to a functional attribute of progenitor cells was tested. The current belief is that the DN1 CD177^{+} (DN1 CD117) population is the most robust T-lineage progenitor and gives rise to T-cells via the DN2 and DN3 intermediates (see Chapter 3). By comprehensively analysing gene expression patterns of respective populations during T-cell development, we aimed to define each stage of development by a molecular signature of that population. Would these global footprints of gene expression allow to understand the relationship between thymic and bone marrow progenitor cells?

The population of cells in the DN1 compartment, which do not express CD117 has never been characterised on the transcriptional level. Here, this fraction of DN1 cells was analysed for expression of genetic elements known to be involved in various aspects of haematopoietic development. Additionally, the DN1 CD117^{-} population was further dissected and the preponderant fraction, the DN1 CD45R population, was analysed for expression of lineage specific genes. With the genetic data obtained from the DN1 CD45R population, would it be possible to infer a developmental potential of these cells and to align it with already well-defined stages of T-cell development?

Results

Gene Expression Analysis of Double Negative Thymocytes

It has been suggested that progenitor T-cells express genes only related to the T-cell lineage and that there is no genetic regulation between DN2 and DN4 stages of thymocyte development (Akashi, He et al. 2003; Hoffmann, Bruno et al. 2003). To address this issue sorted double negative thymocytes (Figure 5.1) were subjected to semi-quantitative RT-PCR interrogating for the expression of various lineage specific genes. Prior to cDNA synthesis, integrity of RNA was assessed on the Agilent BioAnalyzer®. Figure 5.2 shows a representative example of 15 ng RNA from a DN2 population electrophoresed through a NanoChip.

First, genes related to the T-cell lineage were analysed (Figure 5.3A). Expression of GATA-3 was evident at each stage of DN development. Gene expression of Notch-1 was regulated between DN2 and DN4 stages of maturation. Interestingly, expression of this gene segregated in the DN1 population. DN1 CD117- population did not express Notch-1, however, a target gene of Notch signalling, namely pre-Tα, was expressed amongst these cells, even to a higher level than the DN1 CD117+ population, which suggests that the DN1 CD117- population already received a Notch signal. An alternatively spliced form of pre-Tα was detected throughout T-cell development, however, the functional relevance of this isoform is unknown. Expression of CD127 (IL-7Rα) gene correlated to its protein expression.

Next, lymphoid related genes were analysed (Figure 5.3B). There was no detectable expression of Pax-5, however PU.1 was expressed and modulated during thymocyte differentiation, as was the expression of Id2. Expression of CD135 (Flt-3) was only detected in DN1 progenitors.

Finally, myeloid related genes were analysed (Figure 5.3C). Unexpectedly, expression of GMCSF and IL-3 receptor sub-units (GMCSFRα/CD116 and IL3Rα/CD123, respectively) was observed until the DN4 stage of development, whereas expression of ICSBP was abrogated at the DN3 stage of development, thus illustrating genetic promiscuity and genetic differences between various subsets of double negative thymocytes.

Figure 5.1 Sorting Double Negative Thymocytes.

A Representative histograms illustrating the composition of double negative thymocytes from 4 weeks old female C57BL/6 mice as resolved by cell surface expression of CD44 and CD25 before FACS. Cells were isolated based on CD44 and CD25 expression and in addition, the CD44+CD25- DN1 population was dissected according to CD117 expression.

B Reanalysis of DN1 CD117+ population after FACS. Representative histograms illustrate pure DN1 CD117+ population.

C Reanalysis of DN1 CD117- population after FACS. Representative histograms illustrate pure DN1 CD117- population.

D Reanalysis of DN2 (left), DN3 (middle) and DN4 (right) populations after FACS. Representative histograms illustrate good purity of these populations.

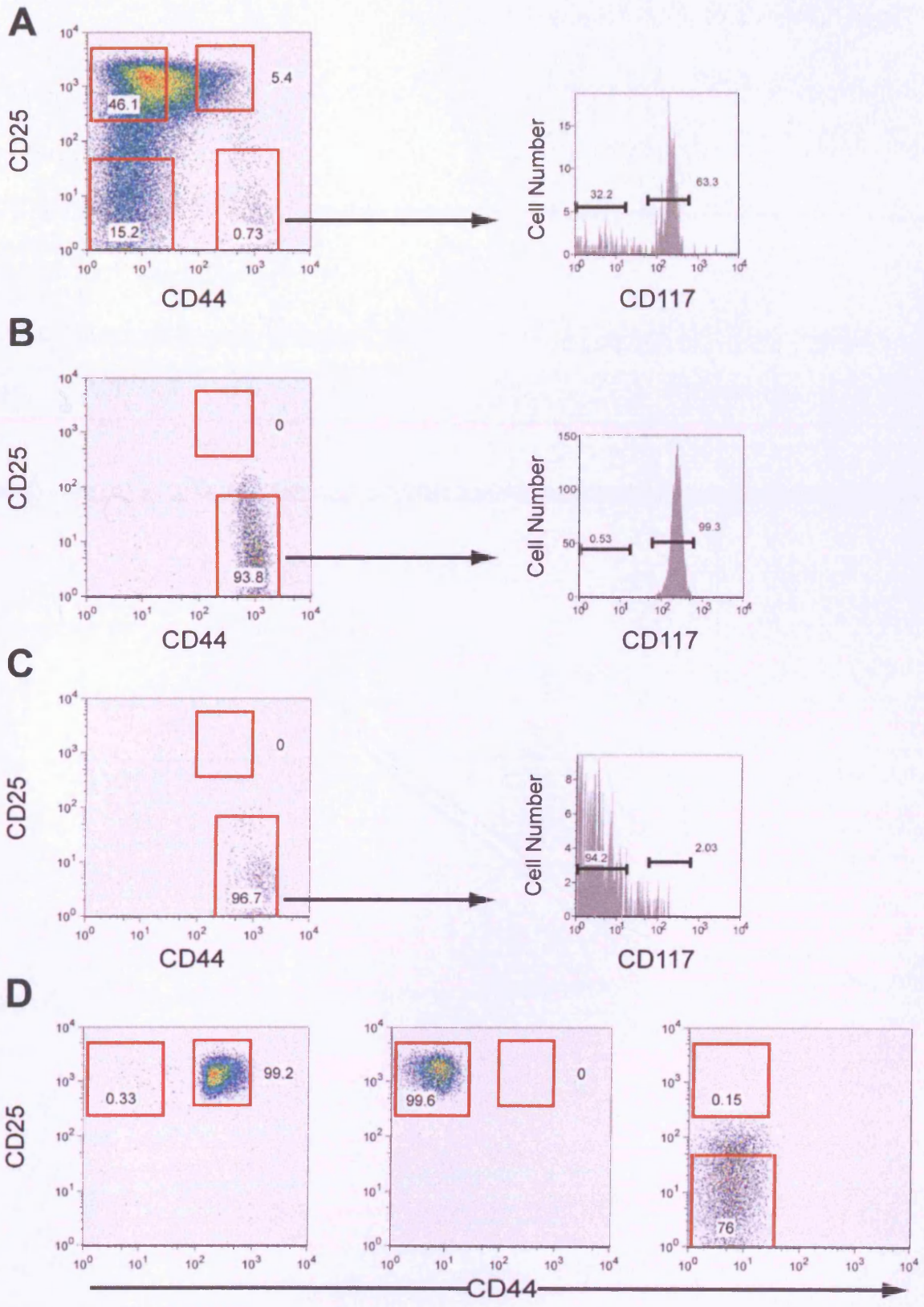

A
CD25
CD44
46.1
5.4
15.2
0.73
Cell Number
CD117
32.2
63.3
B
CD25
CD44
0
93.8
Cell Number
CD117
0.53
99.3
C
CD25
CD44
0
96.7
Cell Number
CD117
94.2
2.03
D
CD25
CD44
0.33
99.2
99.6
0
0.15
76

Figure 5.2 Analysis of RNA Integrity.

Representative RNA profile as assessed by Agilent BioAnalyzer®. 15 ng of total DN2 RNA was fluorescently labelled and electrophoresed on an Agilent NanoChip. Relative fluorescence was detected. Representative histogram shows sharp peaks of ribosomal RNA, which indicates minimal degradation, therefore good quality of RNA. Peak at 40 s represents 18S ribosomal RNA, peak at 46 s represents 28S ribosomal RNA and peak at 23 s is a standard.

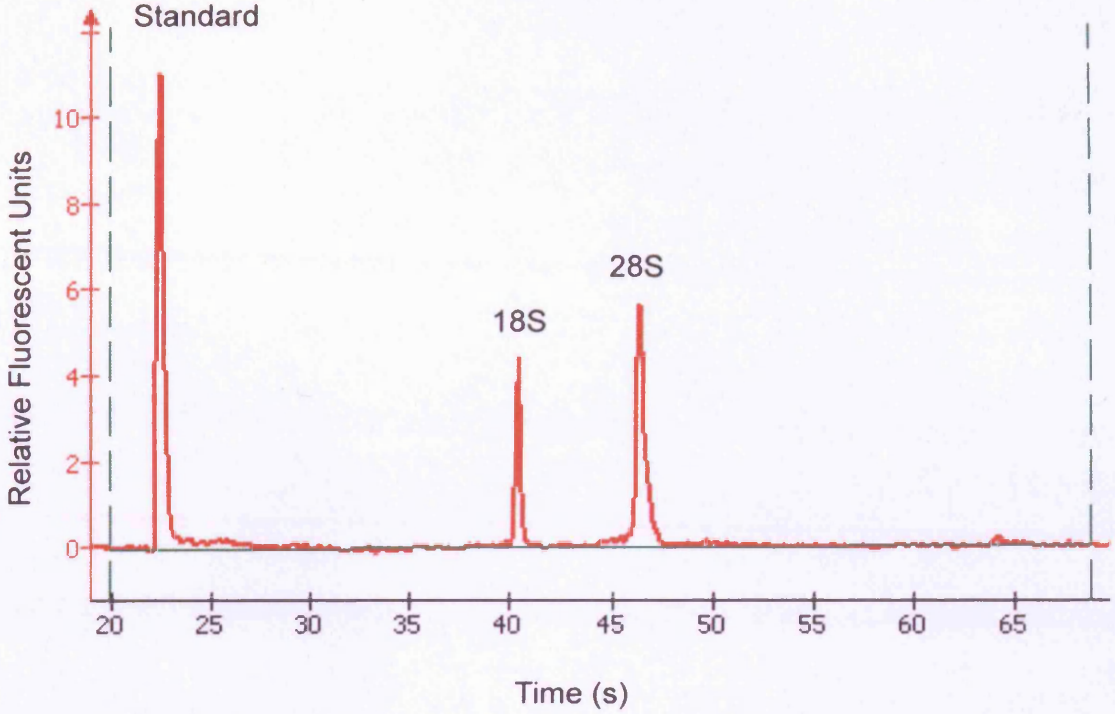

Standard
18S
28S
Relative Fluorescent Units
0
2
4
6
8
10
20
25
30
35
40
45
50
55
60
65
Time (s)

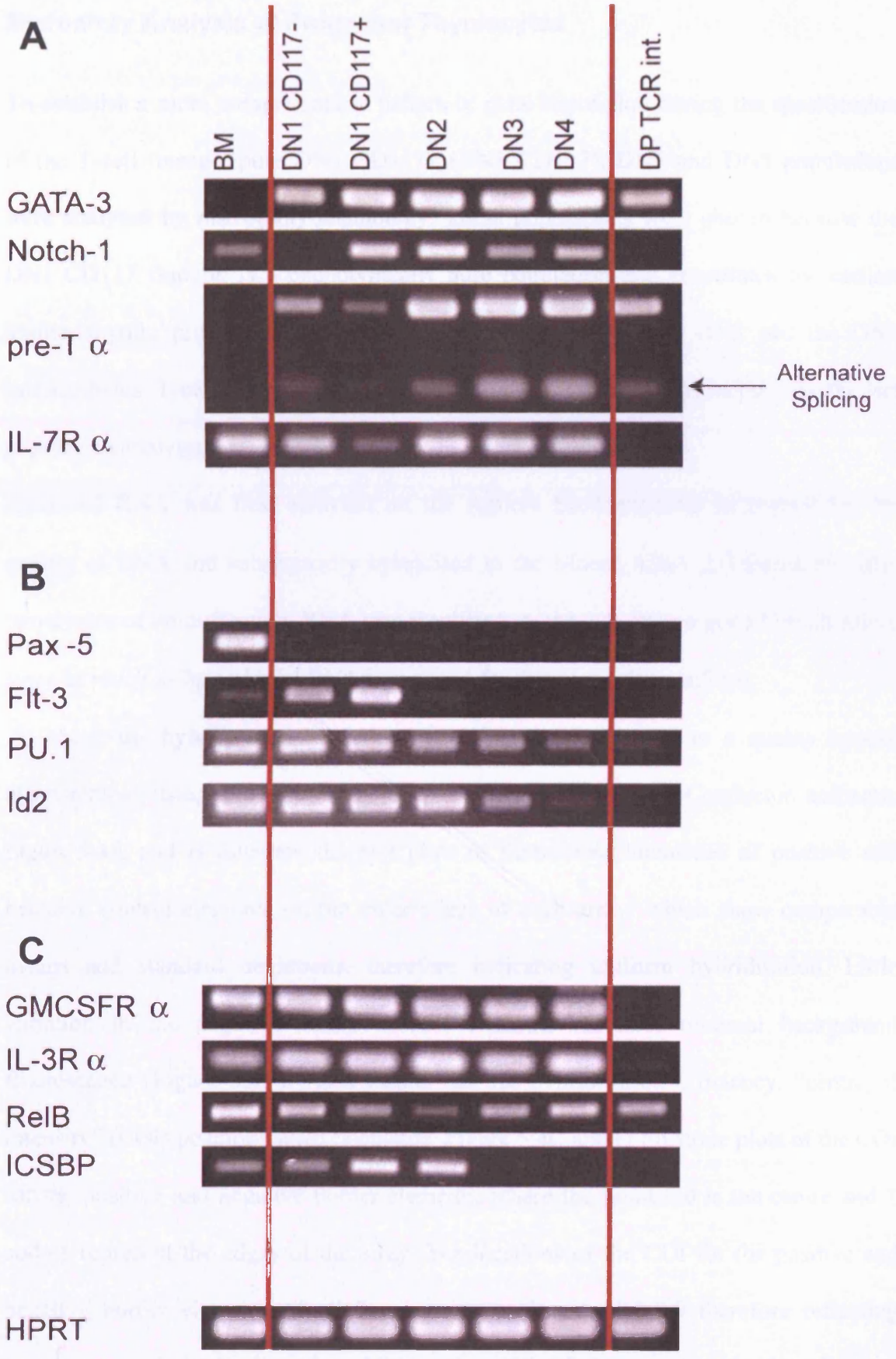
Figure 5.3 Initial Gene Expression Analysis of Developing Thymocytes.

Progenitor populations purified on 5 separate occasions from 4 weeks old female C57BL/6 mice were subjected to semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR), looking at gene expression of elements known to be important in haematopoietic development. Genes were grouped according to their reported lineage affiliation. IL-7Rα - CD127, Flt-3 - CD135, GMCSFRα - CD116, IL-3Rα - CD123.

A Expression patterns of T-cell related genes showed regulation during development. DN1 CD117- and DN1 CD117+ progenitor cells exhibited distinct patterns of gene expression. The smaller pre-Tα product represents an alternatively spliced transcript.

B Expression patterns of lymphoid related genes showed distinct developmental regulation. Pax-5 expression was never detected in thymocyte progenitors. Both DN1 fractions expressed the progenitor cell marker Flt-3 at the genetic level.

C Myeloid related genes showed unexpected expression during T-cell development.


A
BM
DN1 CD117-
DN1 CD117+
DN2
DN3
DN4
DP TCR int.
GATA-3
Notch-1
pre-T α
IL-7R α
Alternative Splicing
B
Pax -5
Flt-3
PU.1
Id2
C
GMCSFR α
IL-3R α
RelB
ICSBP
HPRT

Microarray Analysis of Progenitor Thymocytes

To establish a more comprehensive pattern of gene expression during the specification of the T-cell lineage, pure DN1 CD117+ (DN1 CD117), DN2 and DN3 populations were analysed by microarray technology. These populations were chosen because the DN1 CD117 fraction is a phenotypically pure population and constitutes the earliest known thymic progenitor, which gives rise to T-cells via the DN2 and the DN3 intermediates. T-cell identity is established by the DN3 stage, therefore this was the last population analysed.

Extracted RNA was first analysed on the Agilent BioAnalyzer® to control for the quality of RNA and subsequently hybridised to the Mouse 430A_2.0 GeneChip after two-cycles of amplification. RNA was amplified in order to ensure good hybridisation, since as much as 2μg of total RNA is required for this particular platform.

To check the hybridisation procedure, raw data was subjected to a quality control measurement using the *affyQCReport* package run in the BioConductor software. Figure 5.4A and B illustrate the box plots of fluorescent intensities of positive and negative control elements on the outer edges of each array, which show comparable means and standard deviations, therefore indicating uniform hybridisation. Little variation in the negative control border elements indicates minimal background fluorescence (Figure 5.4B). As a further test for hybridisation efficiency, "centre of intensity" (COI) positions were calculated. Figure 5.4C and D illustrate plots of the COI for the positive and negative border elements, where the point 0.0 is the centre and 1 and -1 represent the edges of the array. The locations of the COI for the positive and negative border elements of all 3 arrays were close to 0.0,0.0 therefore reflecting homogeneous hybridisation. In addition, the COI of the negative border elements illustrated the uniformity of background fluorescence of all 3 arrays (Figure 5.4D).

Raw data was next normalised in GeneSpring GX 7.3 software and analysed in a continuous fashion. Out of 22690 genes represented on the GeneChip, 13907 genes registered a "Present" call in at least one out of three samples and were therefore taken further. Subsequently, 4903 genes exhibited a 1.8 fold regulation at least between two samples, and thus were carried forward for the final analysis. The Pearson correlation algorithm was used to group genes with similar expression levels, and the K-means clustering algorithm was used to group genes with similar expression patterns. Figure 5.5 illustrates the behaviour of genes throughout double negative development and a comprehensive gene list from each cluster is provided on an accompanying data CD.

Figure 5.4 Quality Control of Microarray Experiment.

Quality control measurements of unnormalised data from Affymetrix GeneChip microarray experiments with FACS purified DN1 CD117, DN2 and DN3 progenitor thymocytes from 4 weeks old female C57BL/6 mice, as assessed by the *affyQCReport* package run in the BioConductor software. For each array, the intensities of all border elements were collected. Elements with an intensity > x 1.2 the mean of all border elements were considered to be positive controls and elements with a signal < x 0.8 of the mean were assumed to be negative controls. Elements falling in between these cut offs were not used for further calculations. The 'Centre of Intensity" (COI) positions were calculated by determining the mean fluorescent values for the left, right, top and bottom elements of positive and negative controls. Border elements constituted spiked B2 oligonucleotide, *bio*B, *bio*C, *bio*D and *Cre* oligonucleotides.

A Box plots represent the fluorescent intensity of positive border elements on each individual array. The mean fluorescence, solid black line, is comparable across all 3 arrays indicating homogeneous hybridisation.

B Box plots represent the fluorescent intensity of negative border elements on each individual array. Low values and little fluctuations indicate minimal background fluorescence.

C Each circle denotes the "Centre of Intensity" (COI) position of positive border elements on each individual array. Uniform hybridisation is reflected in the location of each COI close to 0.0,0.0.

D Each circle denotes the "Centre of Intensity" (COI) position of negative border elements on each individual array. There is uniform background across the 3 arrays.

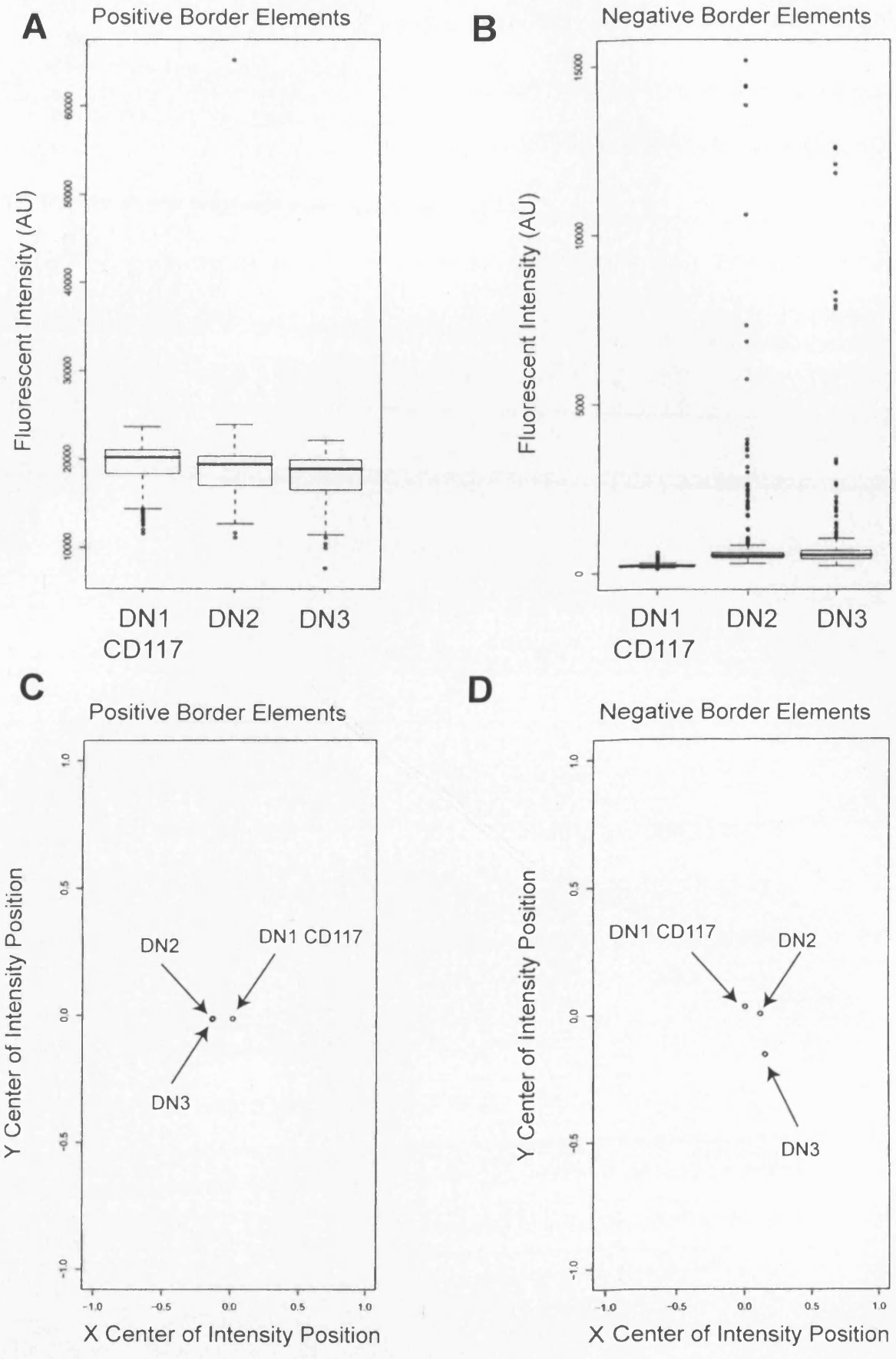
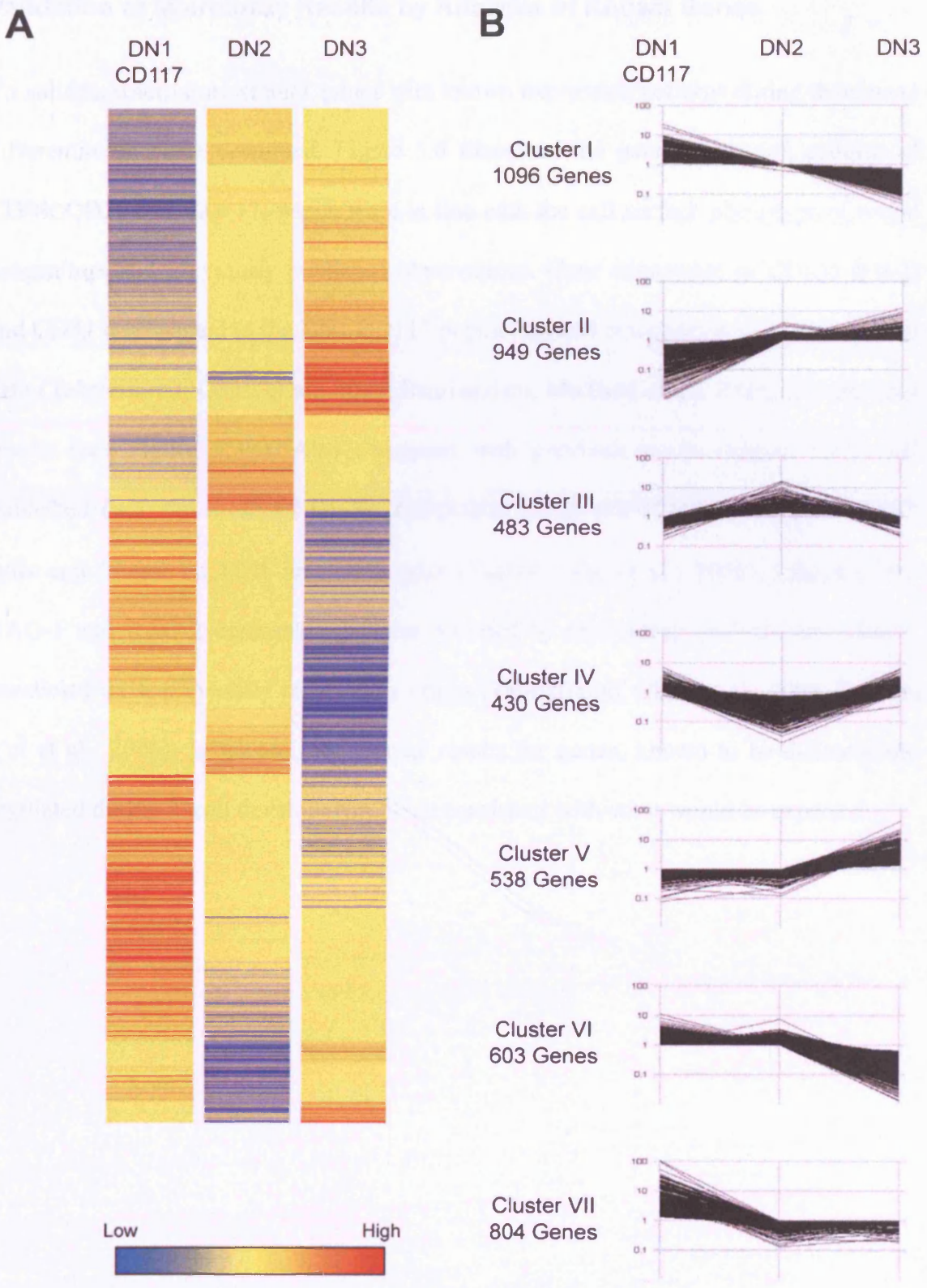

A
Positive Border Elements
Fluorescent Intensity (AU)
10000
20000
30000
40000
50000
60000
DN1 CD117
DN2
DN3
B
Negative Border Elements
Fluorescent Intensity (AU)
0
5000
10000
15000
DN1 CD117
DN2
DN3
C
Positive Border Elements
Y Center of Intensity Position
X Center of Intensity Position
DN2
DN1 CD117
DN3
D
Negative Border Elements
Y Center of Intensity Position
X Center of Intensity Position
DN1 CD117
DN2
DN3

Figure 5.5 Gene Expression Profiling of Double Negative Thymocytes.

Purified DN1 CD117, DN2 and DN3 progenitor thymocytes from 4 weeks old female C57BL/6 mice were transcriptionally profiled on the Affymetrix Mouse 430A_2.0 GeneChip. Raw data was transformed by the MAS 5.0 algorithm run on GCOS and subsequently analysed on GeneSpring microarray analysis software. Genes were filtered on the absolute detection call followed by fold change. Genes exhibiting > x 1.8 fold change between at least 2 samples were classified as regulated and were taken for further analysis.

A Pearson correlation algorithm generated a heat map illustrating clusters of genes with similar expression levels. Each progenitor population has a distinct transcriptional signature.

B K-means clustering revealed 7 distinct gene clusters with different patterns of expression during double negative development.

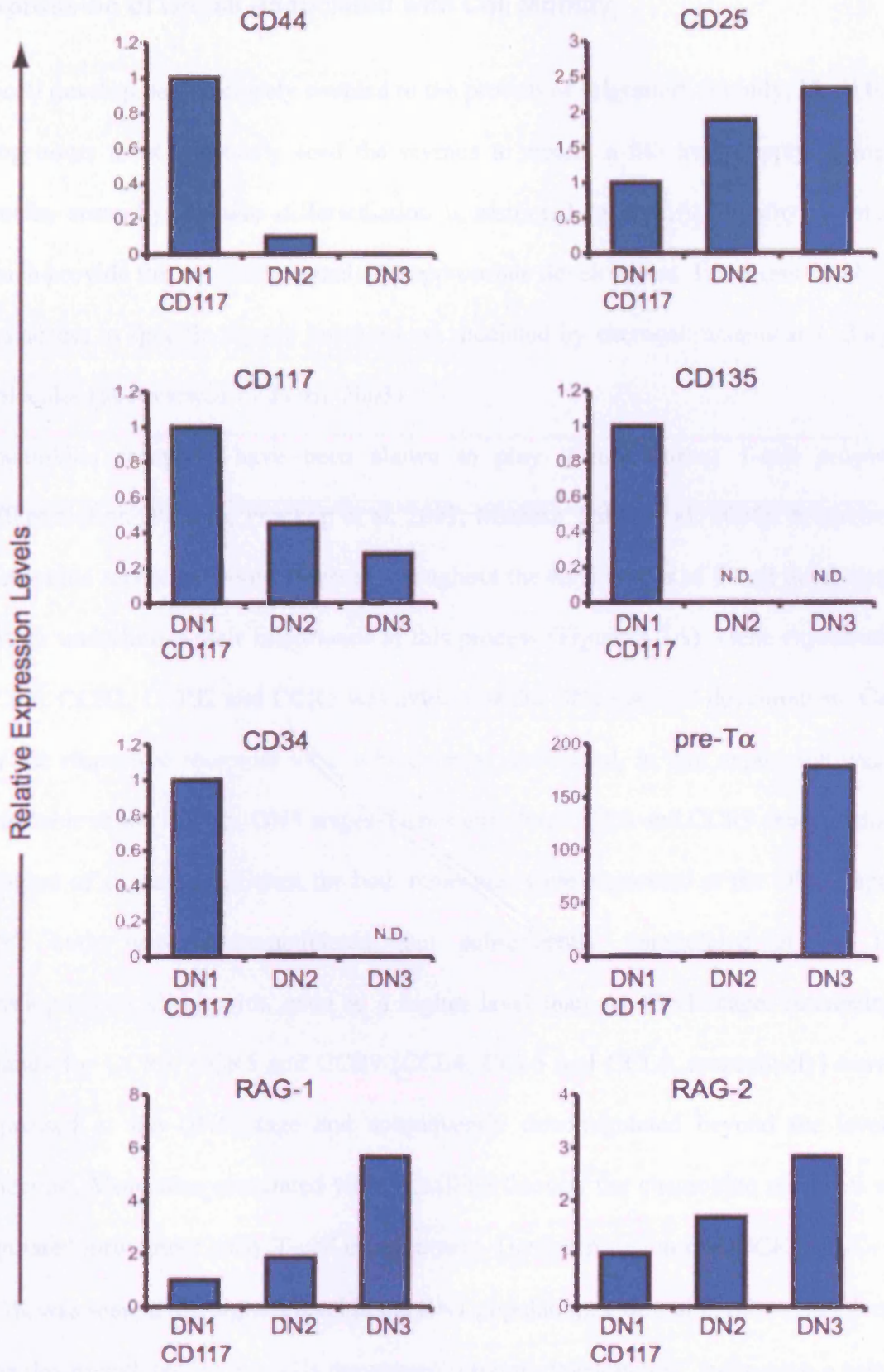

A
DN1
CD117
DN2
DN3
Low
High
B
DN1
CD117
DN2
DN3
Cluster I
1096 Genes
Cluster II
949 Genes
Cluster III
483 Genes
Cluster IV
430 Genes
Cluster V
538 Genes
Cluster VI
603 Genes
Cluster VII
804 Genes

Validation of Microarray Results by Analysis of Known Genes

To validate microarray results, genes with known expression patterns during thymocyte differentiation were examined. Figure 5.6 illustrates the gene expression patterns of CD44, CD25 and CD117, which were in line with the cell surface phenotype of sorted progenitors and previously published observations. Gene expression of CD135 (Flt-3) and CD34 is restricted to the DN1 CD117 population and correlates well with published data (Tabrizifarad, Olaru et al., 2004; Sambandam, Maillard et al., 2005) and previous results (see Figure 5.3B). Also consistent with previous results (Figure 5.3A) and published data, the invariant pre-Tα component of the pre-TCR was highest on DN3 cells and lowest on DN1 and DN2 cells (Taghon, Yui et al., 2006). Likewise, the RAG-1 and RAG-2 expression patterns revealed by microarray analysis very closely paralleled those previously reported by others (Tabrizifarad, Olaru et al., 2004; Taghon, Yui et al., 2006). In general, microarray results for genes, known to be differentially regulated during T-cell development, were consistent with what would be expected.

Figure 5.6 Gene Expression Patterns of Known Developmentally Regulated Genes During T-lineage Specification.

Purified DN1 CD117, DN2 and DN3 progenitor populations were subjected to microarray screening, as detailed earlier. Bar graphs represent gene expression patterns, relative to the DN1 CD117 population, of elements with well-characterised activity during T-cell development. A good correlation is seen between transcript levels of CD44, CD25 and CD117 and respective cell surface expression. Gene expression of CD135 (Flt-3), as assessed by microarray, is in line with previous RT-PCR data.


Relative Expression Levels
CD44
CD25
CD117
CD135
CD34
pre-Tα
RAG-1
RAG-2
DN1 CD117
DN2
DN3
N.D.

Expression of Genes Associated with Cell Motility

T–cell development is closely coupled to the process of migration. Initially, blood borne progenitors must constantly seed the thymus to ensure a life long supply of mature T-cells, secondly, cellular differentiation is restricted to specific intrathymic niches, which provide the necessary signals for appropriate development. Entry into the thymus and access to specific thymic locations are mediated by chemoattractants and adhesion molecules (as reviewed by Petrie 2003).

Chemokine receptors have been shown to play a role during T-cell progenitor differentiation (Plotkin, Prockop et al. 2003; Misslitz, Pabst et al. 2004). A number of chemokine receptors were expressed throughout the early stages of T-cell development, further underlining their importance in this process (Figure 5.7A). Gene expression of CCR1, CCR2, CCRl2 and CCR5 was evident at the DN1 stage of development. Genes for the respective receptors were subsequently inactivated, in that expression was not detectable at the DN2 or DN3 stages. Genes encoding CCR6 and CCR9 showed similar patterns of expression. Genes for both molecules were expressed at the DN1 stage, at DN2 both were downmodulated, but subsequently upregulated at the DN3 developmental checkpoint, even to a higher level than the DN1 stage. Interestingly, ligands for CCR4, CCR5 and CCR9 (CCL4, CCL5 and CCL9, respectively) were all expressed at the DN1 stage and consequently downregulated beyond the level of detection. Molecules associated with signalling through the chemokine receptors were regulated throughout early T-cell development. Gene expression of DOCK2, PLCγ and PI3K was seen at the highest level in the DN1 population, expression of these molecules was downregulated as the cells progressed through development, suggesting a role for chemokine signalling during the earliest stage of T-cell differentiation (Figure 5.7A).

Adhesion to ECM components and endothelia, as well as transmigration, is generally controlled by adhesion molecules. The integrin superfamily of adhesion molecules has been implicated in migration, differentiation and survival of multicellular organisms. Gene disruption of a particular integrin subunit generally leads to a lethal phenotype. The β1 integrin family comprises the largest family of integrins (Figure 1.4). Gene expression of β1 integrin (Itgb1) was upregulated throughout T-cell development, which was mirrored by the pattern of gene expression for one of its partners, α6 integrin (Itga6) (Figure 5.7B). Expression of genes coding for α3 integrin (Itga3) and α4 integrin (Itga4) showed parallel expression patterns, they were expressed at DN1, upregulated at the next developmental stage, but were subsequently undetectable. Another member of the β1 integrin family was regulated throughout T-cell development. α9 integrin (Itga9) was predominantly expressed at the DN1 stage and consequently downmodulated. α4 integrin has an alternative binding partner, β7 integrin (Itgb7) (Figure 1.4) which was exclusively expressed by the DN1 pool. Interestingly, αE integrin (ItgaE), which can only bind β7 integrin, showed an inverse pattern of gene expression, in that it was only detectable at the DN3 checkpoint. Gene expression of β5 integrin (Itgb5) was only observed in the DN1 population and correlated to the pattern of gene expression of its only binding partner αV integrin (ItgaV), which was expressed at DN1, downregulated at DN2 and no longer active by the DN3 developmental point. Expression of the αX integrin (ItgaX) gene was highest in DN1, there was a two-fold downmodulation at the DN2 stage and a complete absence of expression by DN3. This illustrates that specific integrin combinations may be required during distinct stages of T-cell differentiation. Genes associated with integrin mediated signalling, such as Ptk2, Cdc42 and vinculin were regulated during the developmental process (Figure 5.7B). All respective genes were expressed

preponderantly during the DN1 stage and further downregulated as cell matured, illustrating a potential role of integrin mediated signalling at the earliest stage of thymocyte development (Figure 5.7B).

A number of molecules which have the capacity to post-translationally modify integrins, were expressed and showed discrete expression patterns (Figure 5.7C). Matrix metalloproteinase (MMP) 2, 8 and 16 were expressed at the genetic level solely at the DN1 stage and were no longer detected at the next developmental checkpoint. MMP11 was expressed at DN1, upregulated at DN2 and no longer detected at DN3, whereas MMP3 was detected only in the DN3 population, which suggests that integrin activity can be regulated at the protein level during T-cell differentiation.

Gene expression of integrin ligands and co-receptors was noted during T-cell development. Gene expression of ICAM-1 (intercellular adhesion molecule 1) was highest at the DN1 developmental point, with DN2 and DN3 stages showing lower expression levels, whereas VCAM-1 (vascular cell adhesion molecule 1) was only expressed by the DN1 population, suggesting cell to cell interactions between developing thymocytes (Figure 5.7B). Expression of genes coding for the ECM components was detected during T-cell development and showed distinct patterns of expression (Figure 5.7C). Pro collagen type IV alpha5 chain was exclusively expressed by DN3 cells, whereas expression of pro-collagen type IX alpha3 chain was restricted to the DN1 population. Another member of the fibril-associated collagens, pro-collagen type IX alpha1 chain was expressed at DN1, completely undetectable at the DN2 stage and re-expressed at DN3, even to a higher level than the DN1 population, which indicates that developing thymocytes can create their own developmental niche, and in addition facilitate the migration of their developing counterparts.

Apart from integrins, cell-cell adhesion can be mediated by cadherins through homophilic interactions. A number of cadherins were expressed along the T-cell developmental axis and these could be clustered according to their expression patterns (Figure 5.7D). E-cadherin (epithelial cadherin, Cdh1) and PB-cadherin (pituitary and brain cadherin, Cdh22) were expressed by DN1 cells and consequently upregulated during development. LI-cadherin (liver and intestine cadherin, Cdh17) was expressed exclusively by the DN1 population, whereas cadherin-8 (Cdh8) and otocadherin (Cdh23) were expressed only in the DN2 pool, thus implying distinct functional attributes of these adhesion molecules during T-cell development.

Figure 5.7 Gene Expression of Elements Involved in Cell Adhesion and Migration During Early Stages of T-cell Development.

Purified DN1 CD117, DN2 and DN3 progenitor populations from 4 weeks old C57BL/6 mice were transcriptionally profiled by microarray technology as described earlier. Gene expression levels were normalised to expression levels in DN1 CD117. In cases where no expression was detected in DN1 CD117 cells, the subsequent developmental stage, where transcripts were detected, was used as a normalisation control. N.D. – not detected.

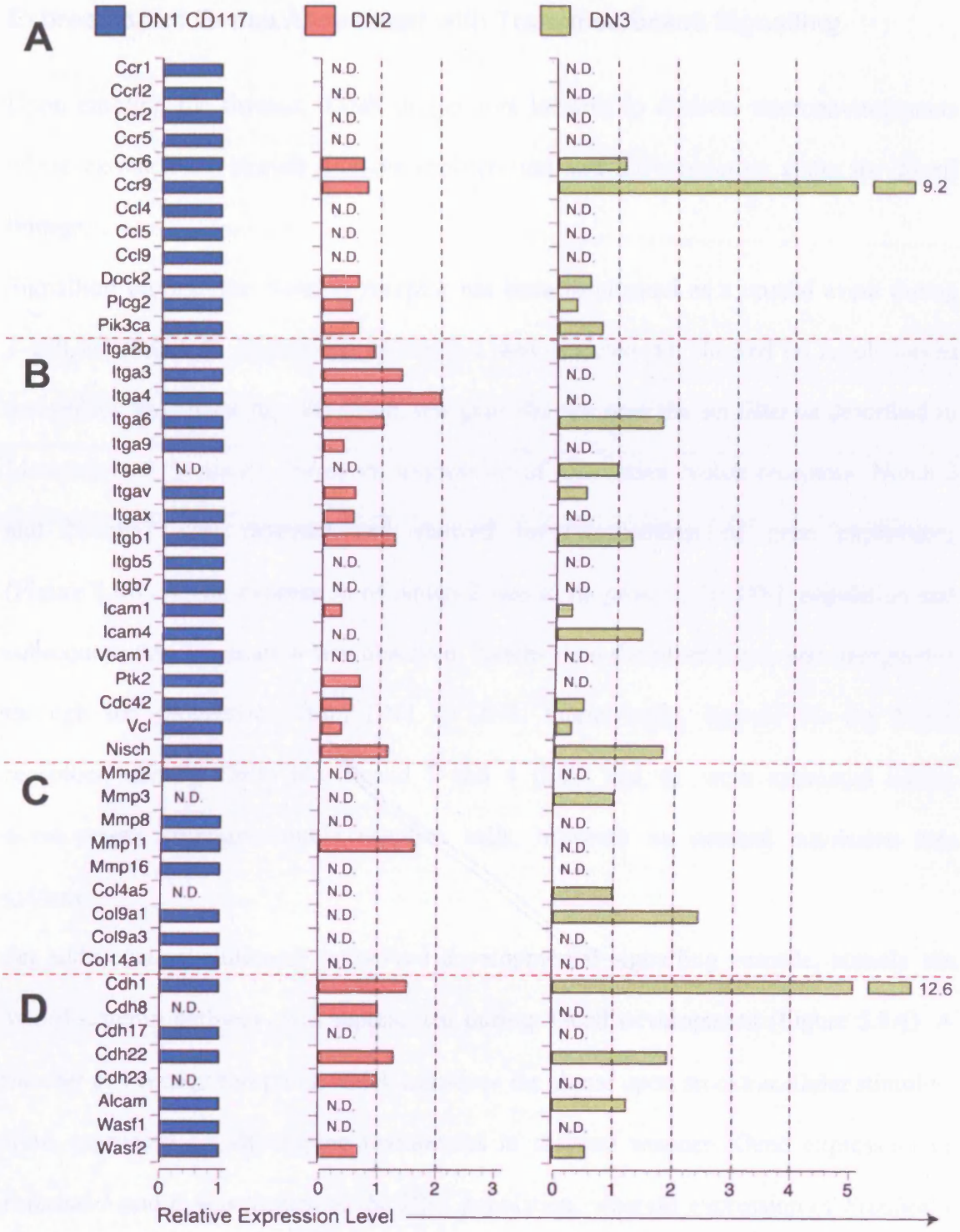
Bar graphs represent relative expression levels of genes associated with cell adhesion and migration.

A Gene expression of chemokines, chemokine receptors and molecules involved in chemokine receptor signalling. A large number of these transcripts are represented in the DN1 CD117 population as compared to later stages of development. A clear exception was the C-C chemokine receptor 9 (Ccr9) which was dramatically upregulated during the early stages of T-cell development.

B Gene expression of integrin receptor subunits, their ligands and molecules involved in integrin mediated signalling. These transcripts exhibited distinct, stage specific regulation. β1 integrin and α6 integrin subunits were upregulated during development.

C Gene expression of extracellular matrix components and molecules involved in integrin modifications. These molecules were expressed predominantly in the DN1 CD117 population.

D Gene expression of alternative adhesion molecules. E-cadherin (Cdh1) was dramatically upregulated during development.


DN1 CD117
DN2
DN3
A
B
C
D
Ccr1
Ccrl2
Ccr2
Ccr5
Ccr6
Ccr9
Ccl4
Ccl5
Ccl9
Dock2
Plcg2
Pik3ca
Itga2b
Itga3
Itga4
Itga6
Itga9
Itgae
Itgav
Itgax
Itgb1
Itgb5
Itgb7
Icam1
Icam4
Vcam1
Ptk2
Cdc42
Vcl
Nisch
Mmp2
Mmp3
Mmp8
Mmp11
Mmp16
Col4a5
Col9a1
Col9a3
Col14a1
Cdh1
Cdh8
Cdh17
Cdh22
Cdh23
Alcam
Wasf1
Wasf2
N.D.
9.2
12.6
0
1
2
3
4
5
Relative Expression Level

Expression of Genes Associated with Transmembrane Signalling

Upon entering the thymus, T-cell progenitors localise to discrete microenvironments where extracellular stimuli promote proliferation and differentiation along the T-cell lineage.

Signalling through the Notch-1 receptor has been implicated as a crucial event during T-cell development. Expression of Notch-1 was detected but showed no regulation as assayed by the GeneChip, therefore, this gene did not pass the set filter as described in Materials and Methods. However, expression of alternative Notch receptors, Notch-2 and Notch-3 was detected and showed inverse patterns of gene expression, (Figure 5.8A). Gene expression of Notch-2 was at its peak in the DN1 population and subsequent downregulation was observed. Notch-3, on the other hand, was upregulated through the progression from DN1 to DN3. Interestingly, ligands for the Notch receptors, namely Delta like ligand 1 and 4 (Dll1 and 4), were expressed during development amongst double negative cells, however no marked regulation was evident.

An additional, evolutionary conserved developmental signalling cascade, namely the Wnt/β–catenin pathway, was represented during T-cell development (Figure 5.8A). A number of Frizzled receptors, which transduce the signal upon an extracellular stimulus, were expressed on developing thymocytes in discrete manner. Gene expression of Frizzled-3 and 6 was limited to the DN1 population, whereas expression of Frizzled-4 was compartmentalised to the DN3 fraction. Gene expression of Frizzled-7 was continuously downregulated during development, whereas Frizzled-2 was expressed by DN1 cells, downmodulated to an undetectable level at the DN2 stage, but re-expressed at DN3, even to a higher level than in DN1 cells. Gene expression of Frizzled receptor ligands was evident during T-cell development. Wnt5b was expressed only by the DN3

pool, whereas Wnt6 was expressed by DN1 cells, it was next downregulated but subsequently upregulated at the DN3 stage, even to a higher level than in DN1, suggesting stage and combination specific requirements of Wnt receptors and their ligands during thymocyte development.

Signalling through receptor tyrosine kinase c-kit (CD117) and Flt-3 (CD135) has been shown to promote T-cell proliferation and differentiation respectively. Since CD135 has been reported as a marker for thymic seeding progenitors and the DN1 population was sorted in addition on its expression of CD117, it was not surprising that both genes were represented in the DN1 population (Figure 5.8C). Gene expression of CD135 was not detected beyond DN1, whereas expression of CD117 was downregulated at the genetic level, mirroring cell surface phenotype. Moreover, expression of the ligand for CD117, stem cell factor (SCF, kit ligand), was detected exclusively within the DN2 population, revealing a potential cell communication axis during early T-cell development.

Signals transmitted through the interleukin-7 receptor (IL-7R) complex are necessary for early thymocyte survival. This requirement is reflected in the upregulation of the IL-7R α (CD127) chain throughout development. The signalling molecule associated with transducing the information from the IL-7 receptor complex, Janus Associated Kinase 3 (JAK-3), was expressed by DN1 cells, but downmodulated subsequently (Figure 5.8B), which suggests that developing thymocytes may store RNA or protein until following developmental stages. A number of other interleukin receptor subunits, such as IL-15Rα and IL-17R, exhibited regulation at the genetic level through various stages of double negative development (Figure 5.8B).

Signalling molecules associated with transmembrane receptor signalling were regulated at the genetic level, implying stage specific functions of these proteins (Figure 5.8D). Molecules associated to the signal transduction through the T-cell receptor (TCR), such

as linker for activation of T-cells (Lat) and lymphocyte specific tyrosine kinase (p56Lck) were already expressed at the DN1 stage and further upregulated until the DN3 stage, whereas Zap70 was expressed only by the DN3 population. Expression of Lck and Lat at DN1 and DN2 stages was surprising, since pre-T-cell receptor signalling is not active until the DN3 stage, which suggests that the products of these genes could be involved in other signalling cascades involving CD45 or interleukin 2 receptor.

The DN1 population expressed a number of genes, which code for growth factor receptors, not previously associated with T-cell development (Figure 5.8C and Table 5.1). Gene expression of macrophage colony stimulating factor 1 receptor (CD115, Csf1r), erythropoietin receptor (Epor), platelet derived growth factor receptor β (CD140b, Pdgfrb) and fibroblast growth factor receptor 2 (Fgfr2) was observed amongst this population, with gene expression downregulated or completely shut off by the subsequent stages of development. In addition, only the DN1 population expressed activin receptor 1 (Alk-2, Acvr1), but bone morphogenic protein 7 (BMP7), which binds Alk-2, was exclusively expressed by the DN3 population, thus once again revealing a potential signalling axis between developing thymocytes.

Figure 5.8 Gene Expression of Elements Involved in Transmembrane Receptor Signalling During Early Stages of T-cell Development.

Purified DN1 CD117, DN2 and DN3 progenitor populations from 4 weeks old C57BL/6 mice were transcriptionally profiled by microarray technology as described earlier. Gene expression levels were normalised to expression levels in DN1 CD117 population. In cases where no expression was detected in DN1 CD117 cells, the subsequent developmental stage, where transcripts were detected, was used as a normalisation control. N.D. – not detected.

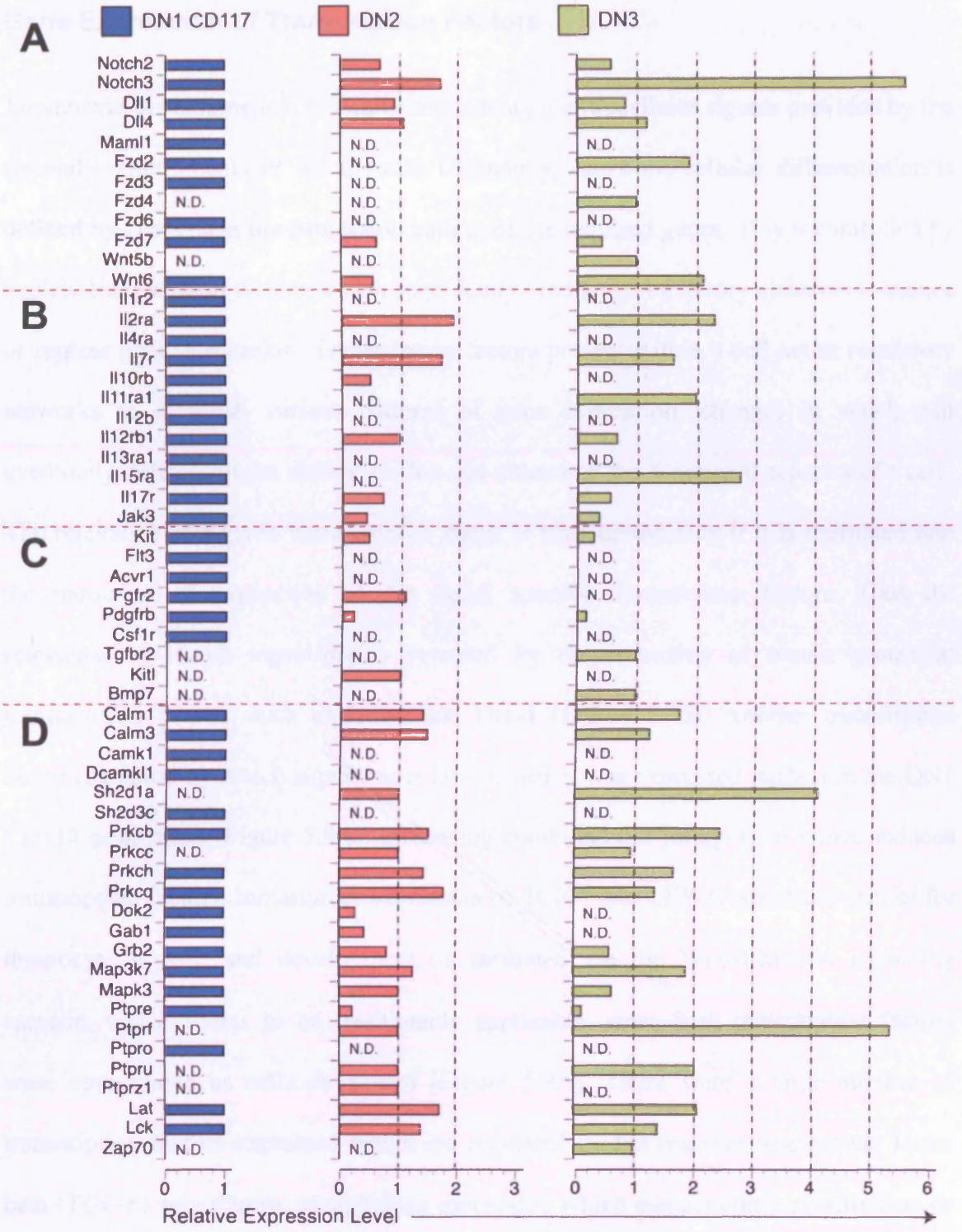
Bar graphs represent relative expression levels of genes associated with transmembrane receptors, their ligands and molecules involved in signalling through these receptors.

A Gene expression of elements involved in Notch and Wnt/β–catenin signal transduction. Notch 3 exhibits marked upregulation during development. Surprisingly, Notch ligands were transcribed but not regulated in developing thymocytes. Expression of Frizzled receptors and their ligands showed stage specific regulation.

B Gene expression of interleukin receptor subunits. The highest expression of most genes is observed in the DN1 CD117 population.

C Gene expression of growth factor receptors and their ligands. The DN1 CD117 population expresses receptors, which are involved in differentiation of a variety of haematopoietic lineages.

D Gene expression of elements involved in signal transduction from transmembrane receptors. These genes show distinct stage specific regulation.


A
B
C
D
DN1 CD117
DN2
DN3
Notch2
Notch3
Dll1
Dll4
Maml1
Fzd2
Fzd3
Fzd4
Fzd6
Fzd7
Wnt5b
Wnt6
Il1r2
Il2ra
Il4ra
Il7r
Il10rb
Il11ra1
Il12b
Il12rb1
Il13ra1
Il15ra
Il17r
Jak3
Kit
Flt3
Acvr1
Fgfr2
Pdgfrb
Csf1r
Tgfbr2
Kitl
Bmp7
Calm1
Calm3
Camk1
Dcamkl1
Sh2d1a
Sh2d3c
Prkcb
Prkcc
Prkch
Prkcq
Dok2
Gab1
Grb2
Map3k7
Mapk3
Ptpre
Ptprf
Ptpro
Ptpru
Ptprz1
Lat
Lck
Zap70
N.D.
0 1
0 1 2 3
0 1 2 3 4 5 6
Relative Expression Level

Gene Expression of Transcription Factors

Thymocyte development is critically dependent on extracellular signals provided by the stromal compartments of the thymus. Ultimately, however, cellular differentiation is defined by changes in the expression pattern of the required genes. This is controlled by nuclear transcription factors, which specifically bind gene regulatory elements to induce or repress gene expression. Transcription factors present within a cell act in regulatory networks to establish various patterns of gene expression, changes in which will eventually define cellular differentiation and ultimately the functional capacity of a cell. The relevance of a given extra-cellular signal is highlighted only if it is translated into the induction of expression of the signal specific transcription factors. Thus the relevance of Notch signalling is revealed by the induction of Notch controlled transcription factors, such as Hes-1 and Dtx-1 (Figure 5.10). Another transcription factor regulated by Notch signalling is Hes-6, which was expressed highest in the DN1 CD117 population (Figure 5.9A), illustrating combinatorial interplay of Notch induced transcription factors. Initiation of expression of TCF-7 and LEF-1, which are crucial for thymocyte growth and development, is mediated via the Wnt/β-catenin signalling cascade, which seems to be functionally applicable, since both transcription factors were upregulated as cells developed (Figure 5.9A). There were a large number of transcription factors expressed which are regulated by the transforming growth factor beta (TGF-β) superfamily of signalling molecules, which can act either as activators or repressors of transcription. There was upregulation of Id-3 throughout the double negative stages, whereas expression of Smad-1, Smad-3 and Smad-4 was predominant at the DN1 stage of development (Figure 5.9B) and mirrored the expression pattern of the associated receptor. A large presence of growth factor receptor genes in the DN1 population was reflected in the expression of the early growth response genes (Egr),

which are targets of these signalling cascades. As a result, Egr1 and Egr2 were expressed to a high level in the DN1 population and were downmodulated at subsequent stages of differentiation (Figure 5.9B).

The establishment of T-cell lineage identity is paralleled with the expression of genes underlining the cells functional potential. The recombinase activating gene (RAG) complex is an essential component in creating a diverse repertoire of antigen receptors, and is controlled, in part, by members of the Runx family of transcription factors. In addition, members of the Runx family of transcription factors are implicated in expression of both CD4 and CD8. Runx-1 was expressed during all stages of double negative development, with levels continuingly increasing up to DN3 (Figure 5.9A). This correlates to the function of Runx-1 as a silencer of the CD4 gene in late DN development. The function of Runx family transcription factors is enhanced by collaboration with partners binding the same regulatory module, such as the Ets family factors. Ets-1 showed a parallel pattern of gene expression to Runx-1 being upregulated from DN1 to DN3 (Figure 5.9A). Other members of the Ets family were expressed amongst double negative thymocytes. GABPα was continuingly upregulated during developmental progression, whereas Fli-1 showed an inverse gene expression pattern (Figure 5.9A) suggesting stage specific combinatorial requirements. A genetic marker of regulatory T-cells is transcription factor FoxP3. There was a notable expression of this gene, along with its family member FoxP1, already at the earliest stages of double negative development (Figure 5.9A) suggesting in initiation of lineage specification even at the earliest stage of T-cell differentiation.

An indispensable transcription factor in T-cell development is GATA-3. This molecule was expressed at basal levels in the populations analysed, therefore, this gene did not overcome the filter specifications described in Materials and Methods. However, other

members of the GATA family were transcribed and regulated at distinct developmental checkpoints (Figure 5.9C). Specifically, GATA-2 and GATA-6 were expressed wholly by the DN1 cells, whereas, GATA-1 and GATA-4 were expressed exclusively by the DN2 population. There is no experimental evidence, which implicates these transcription factors in T-cell development, however, GATA-1 and GATA-2 have been shown to be involved in the development of myeloid lineages. Furthermore, an array of transcription factors which specify alternative haematopoietic lineages were expressed, particularly during the early stages of double negative development (Figure 5.9C). Development of NK-cells is arbitrated by a repressor Id-2 which was expressed by DN1 cells and subsequently downregulated. Gene expression of ICSBP-1, which defines dendritic cells, PU.1, which is necessary for B- and myeloid cell differentiation, and C/EBPβ, required for myeloid development, all showed analogous behaviour to Id-2, being expressed principally by the DN1 compartment, which may reflect the multipotent nature of these progenitor cells. On the other hand, Spi-B, a member of a divergent Ets family, which is necessary for dendritic cell development, was upregulated during the progression from DN1 to DN3, indicating a potential involvement during T-cell lineage development.

Members of the homeodomain-containing HOX genes are important during embryogenesis. A cluster of HOX-A genes was expressed throughout double negative cells, all showing similar patterns of gene activity, with highest expression corresponding to the DN1 population (Figure 5.9D). Constituents of the T-box factor family contribute to lineage specification in a number of developmental systems. Interestingly, T-box 6 and T-box 19 were highly upregulated as the cells advanced from DN1 to DN3 (Figure 5.9D). The Sox (SRY-related HMG box) gene family plays pivotal roles in many aspects of embryonic development. Sox-4, Sox-12 and Sox-17 were all

expressed in early thymic progenitors, being downmodulated accordingly (Figure 5.9D). Conversely, Sox-5 was incessantly upregulated, peaking at DN3, therefore suggesting a potential role in T-cell lineage specification.

Altogether, analysis of transcription factor expression during T-lineage specification illustrated a complex interplay between various regulatory elements involved in establishing T-cell identity. Upregulation of T-cell lineage genes, as a whole, was mirrored in loss of transcription factors implied in establishing or preserving alternative haematopoietic fates, nevertheless, expression of alternative lineage transcription factors during early stages of T-cell development may reflect these cells developmental potential to generate a variety of haematopoietic lineages.

Figure 5.9 Gene Expression of Transcription Factors During Early Stages of T-cell Development.

Purified DN1 CD117, DN2 and DN3 progenitor populations from 4 weeks old C57BL/6 mice were transcriptionally profiled by microarray technology as described earlier. Gene expression levels were normalised to expression levels in DN1 CD117 population. In cases where no expression was detected in DN1 CD117 cells, the subsequent developmental stage, where transcripts were detected, was used as a normalisation control. N.D. – not detected.

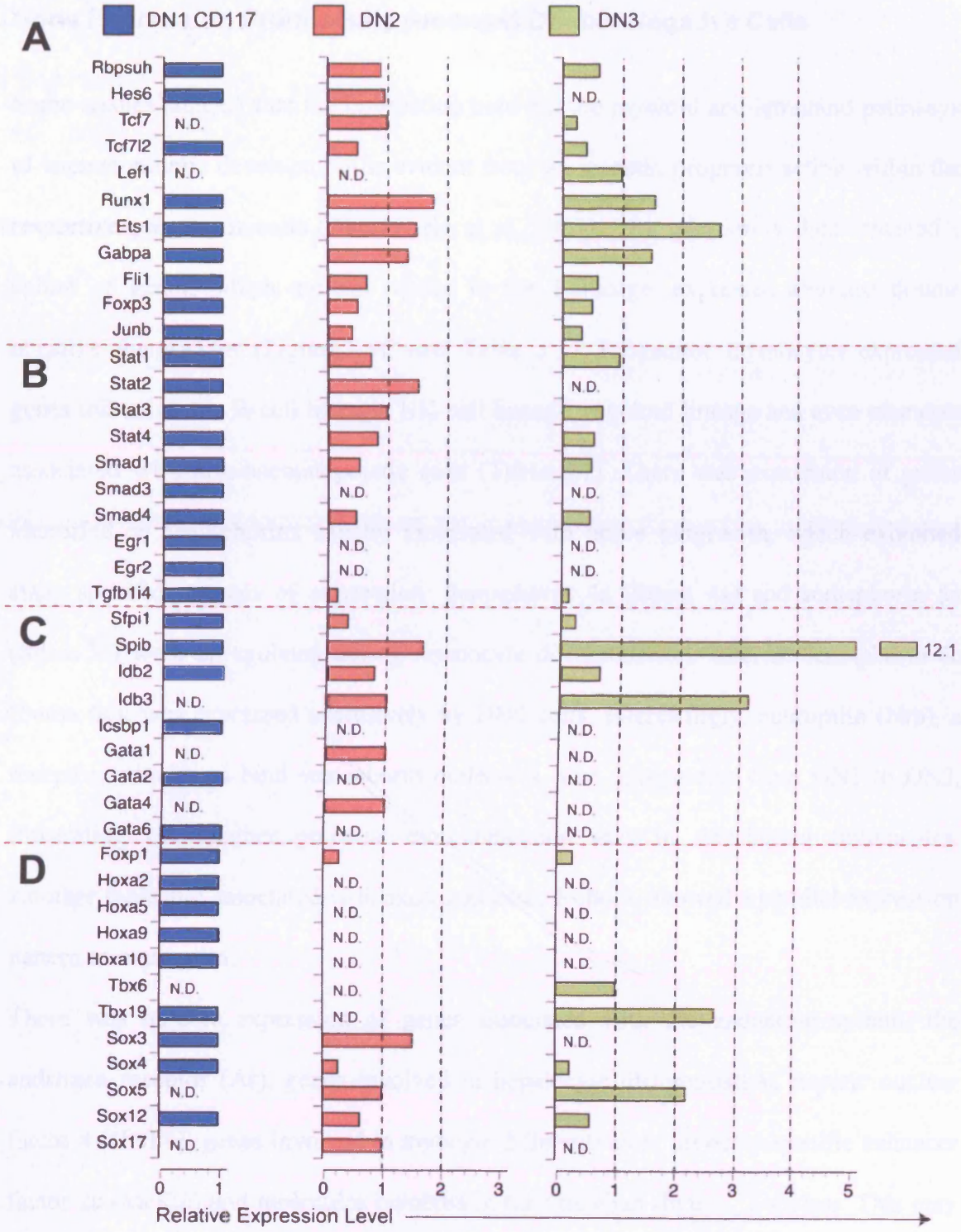
Bar graphs represent relative expression levels of transcription factor genes during early stages of T-cell differentiation.

A Gene expression of transcription factors known to be involved in T-cell development. These show a complex combinatorial interplay during differentiation.

B Gene expression of transcription factors associated with transmembrane signalling. Highest expression of these genes is observed in the DN1 CD117 population, which correlates with expression patterns of relevant cell surface receptors.

C Gene expression of elements involved in specifying alternative haematopoietic lineages. Interestingly, expression is found during thymocyte differentiation.

D Gene expression of transcription factors usually associated with a developmental process. There is a marked upregulation of Sox5 during T-lineage specification.


A
B
C
D
DN1 CD117
DN2
DN3
Rbpsuh
Hes6
Tcf7
Tcf7l2
Lef1
Runx1
Ets1
Gabpa
Fli1
Foxp3
Junb
Stat1
Stat2
Stat3
Stat4
Smad1
Smad3
Smad4
Egr1
Egr2
Tgfb1i4
Sfpi1
Spib
Idb2
Idb3
Icsbp1
Gata1
Gata2
Gata4
Gata6
Foxp1
Hoxa2
Hoxa5
Hoxa9
Hoxa10
Tbx6
Tbx19
Sox3
Sox4
Sox5
Sox12
Sox17
N.D.
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4
5
Relative Expression Level

Gene Expression Promiscuity Amongst Double Negative Cells

Some studies suggest that the bifurcation between the myeloid and lymphoid pathways of haematopoietic development is evident from the genetic programs acting within the respective progenitor cells (Akashi, He et al. 2003). The microarray data revealed a cohort of genes, which are not related to the T-lineage, expressed amongst double negative thymocytes (Figure 5.9C and Table 5.1). Progenitor thymocytes expressed genes related to the B-cell lineage, NK-cell lineage, myeloid lineage and even elements associated with non-haematopoietic cells (Table 5.1). There was expression of genes identified as semaphorins usually associated with nerve outgrowth, which exhibited stage specific patterns of expression. Semaphorin 4a (Sema 4a) and semaphorin 5a (Sema 5a) were upregulated during thymocyte differentiation, whereas semaphorin 6c (Sema 6c), was expressed exclusively by DN2 cells. Interestingly, neuropilin (Nrp), a receptor, which can bind semaphorin molecules, was upregulated from DN1 to DN3, illustrating yet another potential communication axis in developing thymocytes. Another molecule associated with axon guidance, Robo 3, showed a parallel expression pattern to neuropilin.

There was notable expression of genes associated with the endocrine system, the androgen receptor (Ar), genes involved in hepatocyte differentiation, hepatic nuclear factor 4 (HNF4), genes involved in myocyte differentiation, myocyte-specific enhancer factor 2c (Mef2c) and molecules involved in the circadian rhythm, Timeless. This may indicate a potential, as yet, unidentified function of these gene products during T-cell development.

Table 5.1 Promiscuous Gene Expression in Developing Thymocytes.

Table illustrating expression of elements not associated with the T-cell lineage. Expression of alternative haematopoietic lineage genes was evident during early stages of T-cell development. In addition T-cell progenitors expressed genes linked to the nervous, the endocrine and the vascular systems.

	DN1 CD117	DN2	DN3
Ebf1	-	+	-
Syk	++	+	+/-
Cebpb	+	+/-	-
Tlr1	+	-	-
Tlr2	+	-	-
Tlr3	+	-	-
Tlr7	+	-	-
Myd88	++	+	+
Epor	+	-	-
Sema4a	+	++	++
Sema5a	+	+/-	+++
Sema6c	-	+	-
Nrp	+	++	+++
Robo3	+	++	+++
Mef2c	+	-	-
Hnf4	+	-	-
Ar	+	-	-
Vegfa	-	-	+
Vegfb	+	-	-
Gmnn	+	++	+
Qk	++	+	+
Timeless	++	++	+
Sim2	-	-	+

Validation of Microarray Results

In order to test the dynamic range of the microarray platform, and in addition to establish the validity of the microarray platform, expression patterns of genes known to be important in T-cell development were examined by Real-Time PCR and directly compared to microarray results. In addition, the DN1 CD45R population was assessed, since no genetic information is currently available for this progenitor fraction.

Figure 5.10 illustrates expression of genes associated with Notch receptor signalling. Expression patterns of pre-Tα, Hes-1 and Dtx-1 correlated well between the two platforms (Figure 5.10B, C and D). However, expression pattern of Notch-1 showed minor differences. Real-Time PCR analysis revealed that Notch-1 gene expression was continuously upregulated during early stages of development, whereas microarray data revealed no change in gene expression (Figure 5.10A). Gene expression of Notch-1 was upregulated 1.56 ± 0.50 (n = 3) fold in the DN2 population and 1.94 ± 1.2 (n = 3) fold in the DN3 population, as compared to expression in DN1 CD117 cells by Real-Time PCR. This shows only a small modulation, which possibly was out of the dynamic range of microarray technology. A similar limitation could account for differences seen in fold change of GATA-3, Runx-1 and Rag-1 between platforms (Figure 5.11A, B and C). The pattern of expression of the three genes was comparable, however, the regulation range was smaller on the Affymetrix platform, which illustrates that small variations are not readily detectable by microarray. In spite of that, the patterns of gene expression obtained were similar for all genes between the two platforms, therefore validating the microarray results.

The DN1 CD45R population exhibited expression of T-cell lineage genes. Gene expression of Notch-1 was detected amongst these cells and, interestingly, an 8.55 ± 1.68 (n = 3, $p < 0.01$) fold increase in levels of pre-Tα was observed, as

compared to the DN1 CD117 population. This level of pre-Tα gene expression aligns the DN1 CD45R population closer to the DN2 population, $r = 0.97$. Gene expression of Hes-1 and Dtx-1 were minimal in the DN1 CD45R fraction, which suggests that the Notch signal was delivered earlier, perhaps pre-thymically. Gene expression of Rag-1 was 3.77 ± 1.40 ($n = 3$, $p < 0.05$) times higher in the DN1 CD45R population as compared to the DN1 CD117 population, and more similar to the DN2 population ($r = 0.92$), therefore aligning the DN1 CD45R population with the DN2 population. In addition, the DN1 CD45R population expressed comparable levels of Runx-1 and PU.1 genes to the DN1 CD117 population. This complex transcriptional signature positions the DN1 CD45R population between DN1 CD117 and DN2 cells and might indicate that the DN1 CD45R population can serve as an alternative intermediate during T-cell development.

Figure 5.10 Quantitative Analysis of Gene Expression in Double Negative Thymocytes I.

Bar charts represent relative expression of the Notch signalling cascade as assessed by microarray, blue bars, or Real-Time polymerase chain reaction, maroon bars. Determination of expression levels by microarray was described earlier. For Real-Time PCR, purified DN1 CD45R, DN1 CD117, DN2 and DN3 progenitor populations from 4 weeks old C57BL/6 mice were subjected to RNA extraction, quality control and reverse transcription as described in Materials and Methods. 1 x 10^3 cell equivalent cDNA template from each population was amplified in triplicates for every target gene, using the Qiagen primer/probe detection system. Primer/probe sequences are listed in Table 2.2. Expression levels were determined by the regression curve method. Acidic ribosomal phosphoprotein (Arbp) was used as a loading control, subsequently, expression values were normalised to expression in DN1 CD117 population to obtain relative expression levels. Values represent average ± standard deviation from 3 independent preparations of thymic progenitors.

A Gene expression of Notch-1. A modest upregulation was observed during development. The DN1 CD45R population expressed a notable level of Notch-1.

B Gene expression of pre-Tα. This gene showed a dramatic increase during T-cell development. The DN1 CD45R population exhibited higher levels of pre-Tα expression as compared to DN1 CD117 population.

C Gene expression of Hes-1. Little regulation was exhibited during development. Very low expression was observed in the DN1 CD45R population.

D Gene expression of Dtx-1. Highest expression was seen in the DN3 population. The DN1 CD45R showed low levels of this transcript.

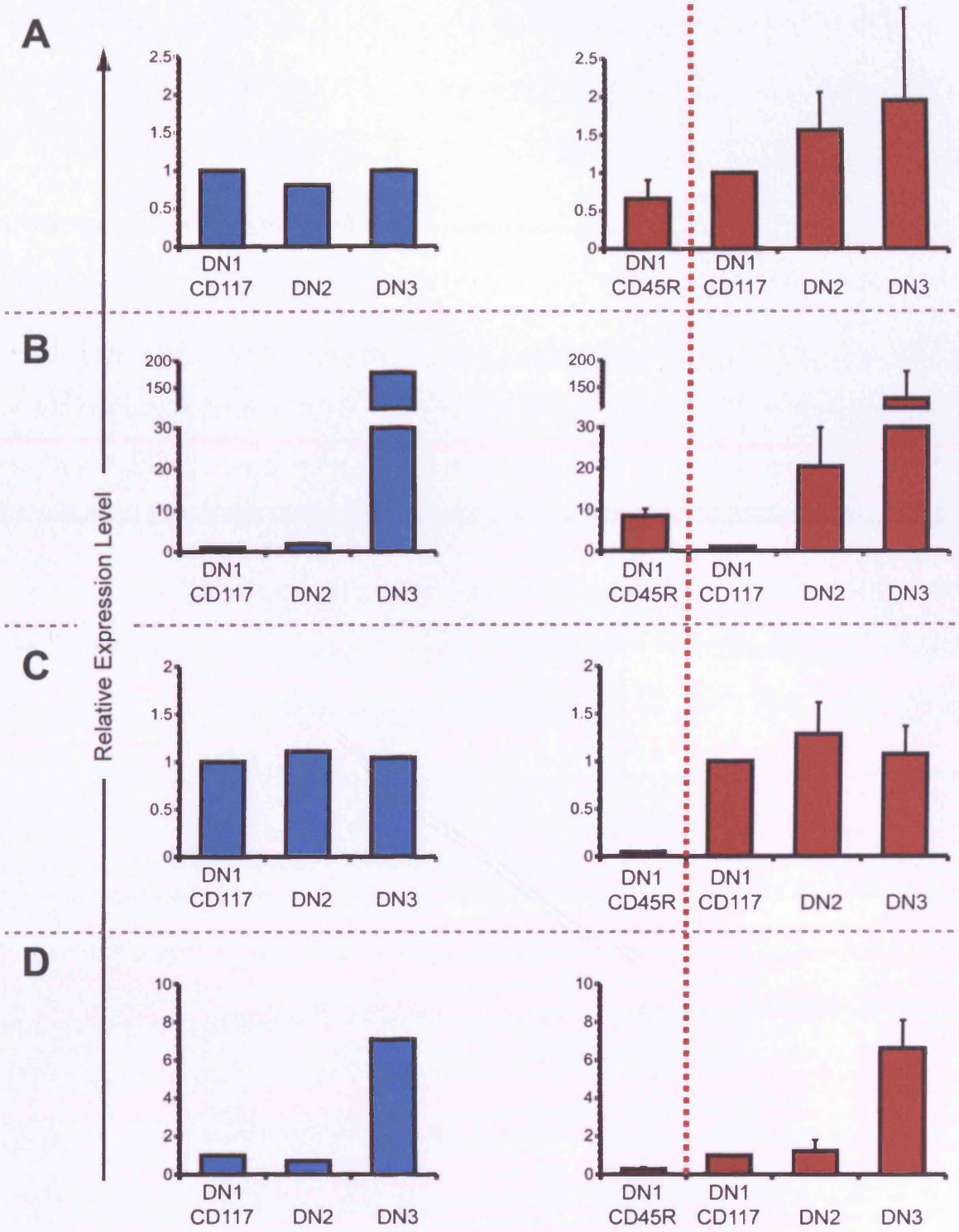

A
B
C
D
Relative Expression Level
DN1 CD117
DN2
DN3
DN1 CD45R
DN1 CD117
DN2
DN3

Figure 5.11 Quantitative Analysis of Gene Expression in Double Negative Thymocytes II.

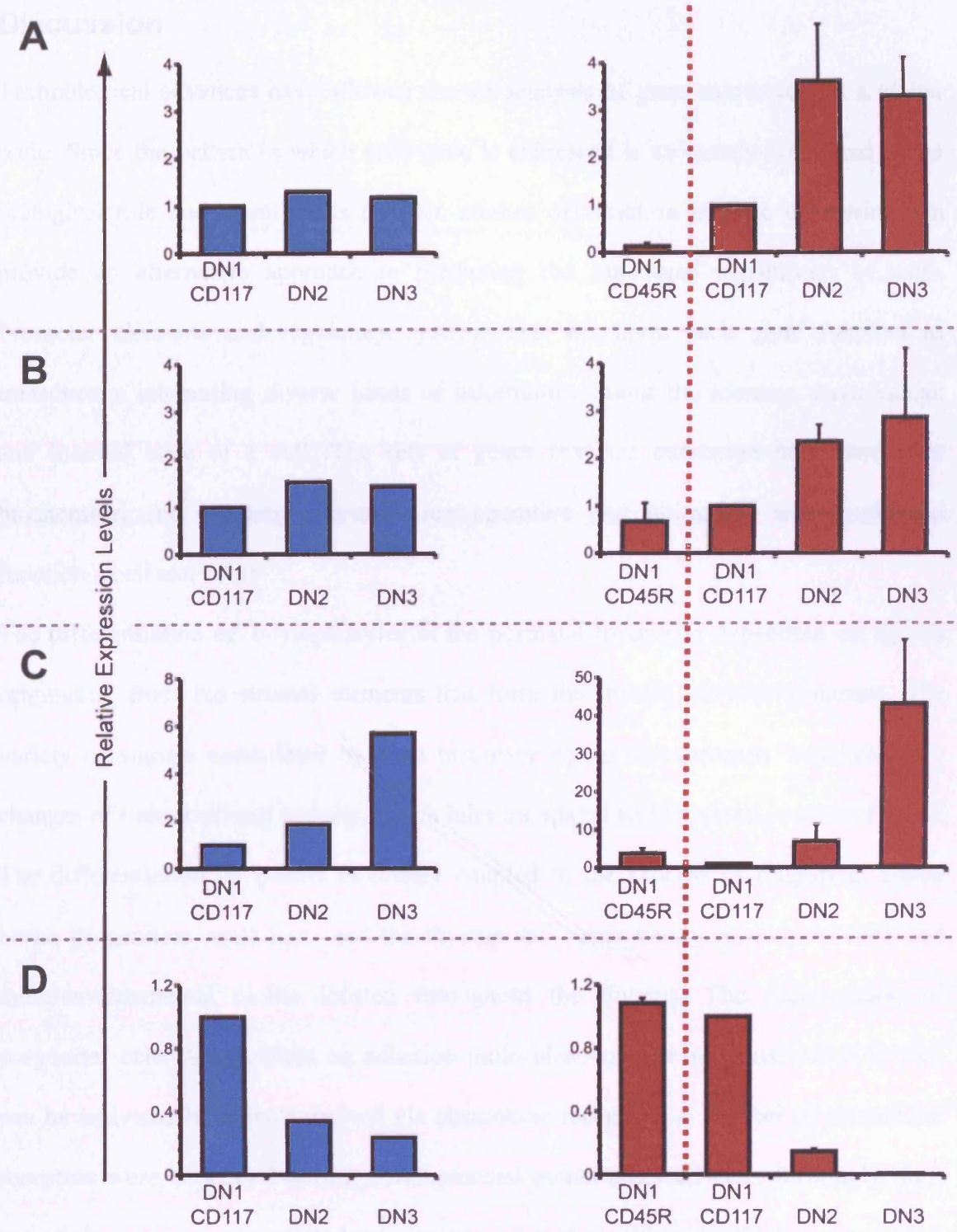
Bar charts represent relative gene expression of elements involved in T-lineage specification as assessed by microarray, blue bars, or Real-Time polymerase chain reaction, maroon bars. Determination of expression levels by microarray was described earlier. For Real-Time PCR, purified DN1 CD45R, DN1 CD117, DN2 and DN3 progenitor populations from 4 weeks old C57BL/6 mice were subjected to RNA extraction, quality control and reverse transcription as described in Materials and Methods. 1 x 10^3 cell equivalent cDNA template from each population was amplified in triplicates for every target gene, using the Qiagen primer/probe detection system. Primer/probe sequences are listed in Table 2.2. Expression levels were determined by the regression curve method. Acidic ribosomal phosphoprotein (Arbp) was used as a loading control, subsequently, expression values were normalised to expression in DN1 CD117 population to obtain relative expression levels. Values represent average ± standard deviation from 3 independent preparations of progenitor thymocytes.

A Gene expression of GATA-3. Expression was highest on DN2 and DN3 cells. The DN1 CD45R population expressed low levels of GATA-3.

B Gene expression of Runx-1. There was upregulation during T-cell development. This gene was expressed in comparable levels in both DN1 populations.

C Gene expression of Rag-1. This gene exhibited a marked upregulation at DN3 stage of development. The DN1 CD45R population exhibited higher levels of Rag-1 in comparison with the DN1 CD117 population.

D Gene expression of PU.1. This transcription factor was downregulated during T-cell commitment. The CD45R population expressed comparable levels with the DN1 CD117 population.


A
B
C
D
Relative Expression Levels
DN1 CD117
DN2
DN3
DN1 CD45R
DN1 CD117
DN2
DN3

Discussion

Technological advances have allowed for the analysis of gene expression on a global scale. Since the pattern in which each gene is expressed is so closely connected to the biological role and effect of its product, studies of variation in gene expression can provide an alternative approach to predicting the functional capabilities of cells. Promoter elements and regulatory systems that act upon each gene function as transducers, integrating diverse kinds of information about the identity, environment and internal state of a cell. The sets of genes that are expressed determine what biochemical and regulatory systems are operative and ultimately what biological function a cell can fulfil.

The differentiation of T-lymphocytes in the postnatal thymus is dependant on signals originating from the stromal elements that form the thymic microenvironment. The variety of signals assimilated by each precursor cell is subsequently translated into changes of transcriptional activity, which infer the spatial and temporal context of a cell. The differentiation of T-cells is closely coupled to the process of migration. Blood borne progenitors must first seed the thymus and subsequently develop in restricted microenvironmental niches located throughout the thymus. The translocation of progenitor cells is dependant on adhesion molecules, such as integrins, which in turn can be activated by signals derived via chemokine receptors. A number of chemokine receptors were expressed during developmental points analysed here. Strikingly, four out of six receptors represented were expressed exclusively in the DN1 compartment, suggesting that this population of cells has been or is responding to chemokine gradients, which are necessary for thymic entry. Upregulation of CCR9 during development implies a possible role of this receptor in early stages of T-cell development, which is corroborated by the finding that cells expressing CCR9

constitute the thymic multipotent progenitor population (TMP). However, a mutation in the *Ccr9* gene did not reveal any dramatic abnormalities in T-cell development (Wurbel, Malissen et al. 2001), suggesting compensatory mechanisms in place in developing thymocytes.

Integrin receptors and cadherins were expressed throughout T-cell development, showing a stage specific pattern of gene expression, underlining the migratory properties of developing thymocytes. There was a dramatic upregulation of E-cadherin during thymocyte differentiation, which is in line with reported data (Lee, Sharrow et al. 1994), therefore suggesting a potential role of this molecule in T-cell differentiation, possibly as an additional regulator of the Wnt/β-catenin signalling cascade (Orsulic, Huber et al. 1999). α4 integrin has been implicated in development of T-cells (Arroyo, Yang et al. 1996; Arroyo, Taverna et al. 2000). The functional readout is consistent with the genetic data, which illustrates expression of this subunit at early stages of T-cell development. The binding partner of α4 integrin, β1 integrin, is expressed throughout thymocyte differentiation, which reflects the functional requirement of this molecule, since β1 integrin is necessary for homing of haematopoietic progenitors to primary lymphoid organs (Potocnik, Brakebusch et al. 2000). Cell surface expression of integrin subunits has previously been reported (Prockop, Palencia et al. 2002). Gene expression of α6 integrin and β1 integrin closely correlated to the cell surface phenotype of developing thymocytes. However, there are discrepancies between the genetic data and the protein expression data for the α4 integrin and α5 integrin subunits. On the surface, both integrin subunits were expressed during the DN1-3 stages of differentiation. At the genetic level, α4 integrin was expressed until DN2 only, whereas α5 integrin was not at all expressed. This suggests an additional post-translational regulation mechanisms

involved in the maintenance of these adhesion molecules, which was reflected in gene expression of matrix metalloproteases during thymocyte differentiation.

Cellular proliferation and quiescence are tightly regulated throughout the T-cell developmental progression. Proliferation is closely coupled to the process of differentiation, therefore, some DN1 and DN2 progenitors are actively dividing (see Chapter 3). The DN3 compartment is quiescent (see Chapter 3 and Hoffman, Passoni et al. 1996). These cells are in the process of TCR β-selection; only those cells, which have successfully rearranged the TCR β chain, receive a signal for further division and differentiation. The proliferative capacity of developing thymocytes can be predicted by the pattern of gene expression of elements responsible for both, cell growth and dormancy. The DN1 population is rich in transcripts coding for cell surface receptors, which transmit growth signals into the cell. These include receptors for the Wnt/β-catenin signalling pathway, receptor subunits specific for various interleukins and protein tyrosine kinase receptors. Gene expression of c-kit (CD117) and flt-3 (CD135) was highest in the DN1 population, which correlates to the cell surface phenotype of these cells. The ligands of these receptors, stem cell factor (SCF, KitL) and flt-3 ligand (Flt-3L) have been implicated in growth and proliferation of early thymocytes (Moore and Zlotnik 1997), and are routinely used for *in vitro* expansion of these cells. Surprisingly, gene expression of SCF was observed amongst the DN2 population of cells, therefore generating autocrine and paracrine circuitry and additionally regulating cell proliferation independent of the stromal elements. The phenomenon of feedback loops amongst developing thymocytes is also illustrated by another signalling axis, the Alk-2/BMP7 pathway. The activin receptor 1 (Acvr1, Alk-2) was exclusively expressed by the DN1 population, whereas its ligand, BMP-7, was expressed by the DN3 pool only, thus generating crosstalk between the two

populations. Noteworthy is the effect of this signalling cascade, which would lead to a probable arrest in differentiation of the DN1 population, however this has not been directly shown in T-cell progenitors. An arrest at the DN1 developmental stage is seen in foetal thymic organ cultures treated with fibroblast growth factors 7 and 10 (FGF7 and FGF10 respectively) (Tsai, Lee et al. 2003). The gene expression profiling demonstrated that the receptor of these growth factors, FGF receptor 2 (FGFR2), was expressed by the DN1 cells, which corroborates the functional data and demonstrates an intricate mechanism in place, to inhibit unnecessary differentiation of progenitor cells, due to a lack of downstream environmental niches, for example. This illustrates the existence of a tight balance between proliferation and differentiation amongst the earliest thymocyte population to achieve maximum efficiency from this pool. On one hand, amplification is required to sustain more mature stages of development, but on the other, systems are available to prevent creation of bottlenecks within the developmental pathway. The sessile nature of DN3 cells is reflected in expression of genes, products of which are usually associated with an antiproliferative signal. This is mirrored in expression of the transforming growth factor β receptor type 2 (TGFBR2) by the DN3 cells and a dramatic upregulation of protein tyrosine phosphatase receptors (PTPR). Among these, PTPRF (LAR) has been shown as a T-cell specific marker, which is upregulated upon thymocyte differentiation (Terszowski, Jankowski et al. 2001).

Lineage specific transcription factors have been identified within the haematopoietic system, which establish the specification of the respective lineage. The best example is Pax-5 in securing the B-cell identity (Nutt, Heavey et al 1999). *Pax-5* null- pro-B cells are uncommitted and are able to differentiate along the myelomonocyitic lineage. For the myeloid lineage, C/EBPα has been identified as a switch, inducing myeloid differentiation from multipotent progenitor cells (Suh, Gooya et al. 2006). Such factor

has so far been elusive for the T-cell lineage. GATA-3 is an essential factor for the initial stages of T-cell development (Ting, Olson et al. 1996), however progenitor cells expressing this molecule also have the capacity to generate alternative haematopoietic lineages. Notch-1 signalling seems to play a vital role in T-lineage specification, however, this signalling mechanism is not exclusive to T-cells, it is also required for the maintenance of the haematopoietic stem cell pool. The products of Wnt/β–catenin signalling TCF-7 and Lef-1 are necessary for T-cell differentiation, however these molecules are also expressed in a number of other systems. The requirement of Notch-1 signalling and Wnt/β-catenin signalling is highlighted by gradual upregulation of Notch-1 as well as its targets, Dtx-1, Hes-1 and pre-Tα, as well as TCF-7 and Lef-1. Interestingly, Notch-2 and Notch-3 were expressed throughout T-cell development, but their functional relevance is unknown. It would seem improbable for these receptors to play identical or compensatory roles to Notch-1, since in the absence of Notch-1, T-cell development is completely abrogated.

The microarray data provides a plethora of candidate genes, which can fulfil the role of a deterministic transcription factor in the T-cell lineage. The Sox family of transcription factors play diverse roles during development of an organism. Their common feature is an SRY-related HMG box domain, which can potentially facilitate assembly of cell specific transcriptional complexes by inducing a bend on the DNA helix (reviewed by Pevny and Lovell-Badge 1997). Five members of the Sox family were expressed amongst double negative thymocytes, showing distinct expression patterns. Of these, Sox5 was upregulated in the DN3 population, a stage of irreversible commitment to the T-cell lineage. Functional Sox5 is required for the generation of the vertebrate skeleton (Smits, Dy et al. 2004; Smits, Lefebvre 2003). Upregulation of this transcription factor in DN3 cells suggests a potential role of this molecule during T-cell differentiation.

Similar induction in expression was observed for T-box 6 transcription factor. This molecule is a member of the T-box family of transcription factors which are defined by a conserved sequence coding for the T-domain involved in DNA binding and protein dimerisation (reviewed in Minguillon and Logan 2003). T-box 6 has been implicated in paraxial mesoderm formation (Chapman, Agulnik et al. 1996) and is interestingly a target of Alk-2 signalling. Even though the gene expression pattern of all these transcription factors suggests their potential involvement in T-cell development, functional studies would be required to further understand the role of these molecules in thymocyte differentiation.

Development of T-cells is initiated in the bone marrow of an adult organism from a multipotent haematopoietic stem cell capable of self-renewal. Previously published reports have suggested a gradual filtration of genetic programs as cells become more committed to either the lymphoid or the myeloid pathways of the haematopoietic system (Akashi, He et al. 2003). This would indicate that thymic progenitor cells are limited in the diversity of expressed genes, strongly biasing the genetic program associated with events taking place during T-cell differentiation. In part this is true, and was mirrored in induction of genes controlled by signalling through the Notch-1 receptor, upregulation of genes responsible for the recombination of the variable receptor (Runx-1, Rag-1, Rag-2) and initiation of gene expression of factors required for thymocyte proliferation (Lef-1, TCF-7). In addition, thymocyte development was accompanied by upregulation of inhibitor of differentiation 3 (Id3), which is vital for the generation of γ/δ T-cells. However, microarray analysis revealed that progenitor thymocytes were not restricted solely to the T-cell specific genetic program. Transcription factors implicated in alternative haematopoietic cell fate determinations were expressed at various levels amongst double negative progenitors. Development of

NK-cells is dependent on a basic helix-loop-helix transcription factor Id2 (inhibitor of DNA binding 2) (Yokota, Mansouri et al. 1999). The ability to generate NK cells by early thymocytes is well established. This functional attribute is reflected in the expression of Id2 by the earliest thymic progenitor, DN1 CD117. As cells develop along the T-cell axis the competence to generate NK cells is diminished in DN2 and completely lost by the DN3 stage. Gene expression of Id2 parallels the functional ability of cells, gradually being downregulated from DN1 to DN3. A similar pattern was observed for genes specifying dendritic cell lineages, RelB and ICSBP (Aliberti, Schulz et al. 2003). Expression of these genes was drastically reduced once the cells competence to generate DCs diminished. The ability to generate B-cells by early thymocytes is a disputed topic. There was no detectable expression of Pax-5 at any stage of thymocyte development, however, Sox4 and PU.1, molecules necessary for B-cell development (Schilham, Oosterwegel et al.1996; DeKoter, Singh 2000) were expressed highest in DN1 cells, being downregulated as the cells lost the ability to form B-cell progeny. Transcription factor PU.1 is also involved in myeloid development (DeKoter, Singh 2000) and complementary to C/EBPβ, illustrated a pattern of gene expression analogous to the functional potential of progenitor thymocytes. Moreover, expression of cell surface receptors associated with the myeloid and erythroid lineages, colony stimulating factor 1 receptor (csfr1) and erythropoietin receptor (EpoR) respectively, were detected at the DN1 stage of development. This suggests two possibilities. Either, the early thymic progenitors are multipotent in their functional attributes at the single cell level, and sequentially lose the ability to generate alternative haematopoietic lineages therefore downmodulating the respective lineage affiliated genes. Alternatively, the DN1 CD117 is heterogeneous and comprises different progenitor populations with discrete functional attributes, which express distinct genetic

programs. For subsequent differentiation, only a fraction of cells is selected, therefore the functional potential along with the transcriptional activity become filtered. Gene expression studies at the single cell level would facilitate in resolving this issue.

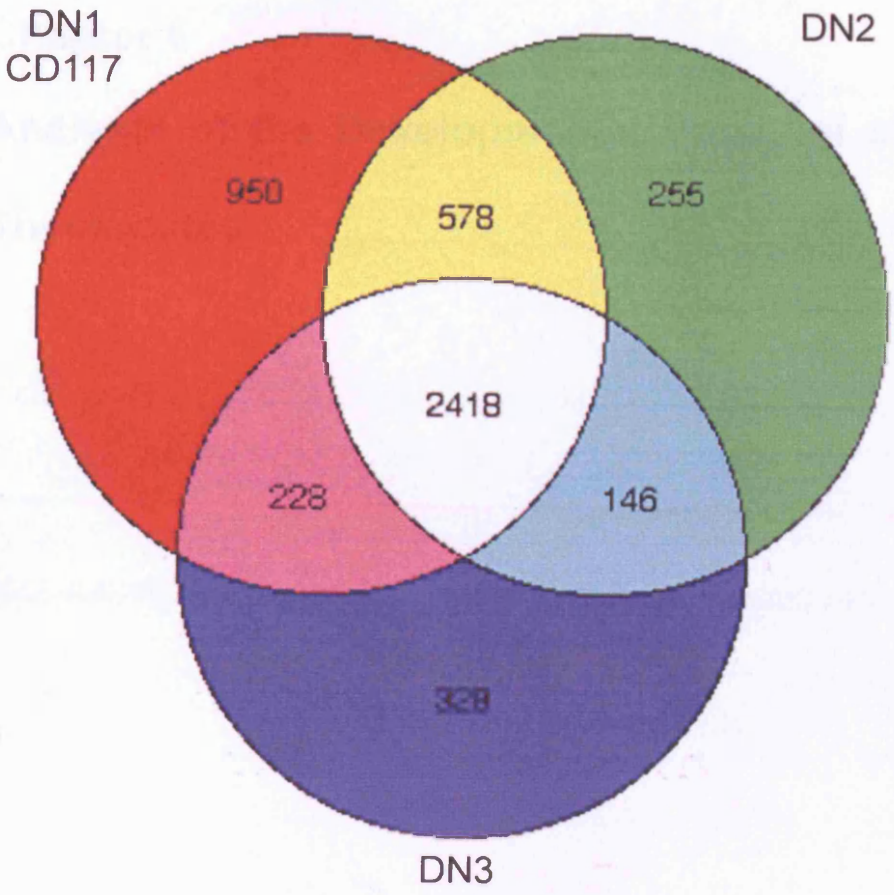
Promiscuous expression of genes observed during double negative development was not only restricted to elements associated with the haematopoietic system. Gene expression of factors linked to the nervous, the endocrine and the vascular systems were well represented throughout the differentiation stages examined. The similarity between certain aspects of the nervous system and the haematopoietic system development (Veiga-Fernandes, Coles et al. 2007) as well as the transdifferentiation potential of the haematopoietic stem cells into various non-haematopoietic lineages (Lagasse, Connors et al. 2000; Krause, Theise et al. 2001) may suggest a functional relevance of these transcripts. Genetic programs operating in HSCs, which constitute non-haematopoietic cell fates may not be fully extinguished, remnants of which are visible in developing thymocytes. However, the unexpected potential of haematopoietic stem cells to generate non-haematopoietic cell types is widely disputed (Wagers, Sherwood et al.2002), therefore an alternative explanation for promiscuous gene expression of non-haematopoietic elements could be the nuclear microenvironment surrounding these loci. Transcription would be initiated due to the open chromatin conformation arising from activity of flanking genetic elements and presence of factors responsible for gene expression, which can be shared by some regulatory systems. Ultimately, the non-haematopoietic genes may have as yet an unidentified obligation in T-cell development.

Global gene expression profiling of developing thymocytes has generated a molecular signature of each progenitor population. Figure 5.12 illustrates that each double negative population has a distinct transcriptional footprint, which can classify every stage of development. These molecular definitions serve as a baseline of genetic events

taking place during T-lineage commitment. Newly identified populations can be interrogated for the expression of genes relating to each cluster and thus aligned and inserted into the genetic scheme of T-cell development. This idea was partially tested on the DN1 CD45R population, a population in the adult thymus which has not been previously characterised. These cells expressed a number of T-lineage affiliated genes, some even to a higher degree than the "canonical" T-cell progenitor, the DN1 CD117 population. Expression levels of pre-Tα and Rag-1 would align the DN1 CD45R population with DN2, therefore suggesting their commitment status as biased to the T-lineage, whereas, expression of PU.1 and Runx-1 would align these cells with the DN1 CD117 population, thus implying their status as early progenitors. This complex pattern of gene expression in the DN1 CD45R population would suggest that these cells might harbour the developmental potential to generate T-cells, thus implying an alternative intermediate in T-cell development.

Figure 5.12 Transcriptional Definitions of Early Thymic Progenitors.

Each double negative progenitor population generated a molecular signature, which allowed the definition of a specific developmental stage based on the transcriptome. If these are aligned and compared, then a preponderant number of genes are revealed, which co-express in all 3 populations, and probably relate to the general homeostasis of a cell. Genes were co-expressed between only 2 populations, therefore revealing intermediate stages during the developmental process, but ultimately, each progenitor population exhibited a specific transcriptional signature. This can now be used to identify each population at the molecular level.


DN1
CD117
DN2
950
578
255
2418
228
146
328
DN3

Chapter 6

Analysis of the Developmental Potential of Progenitor Thymocytes

Introduction

The thymus is a primary lymphoid organ capable of generating all T-cells. The organ itself is unable to produce precursor cells which give rise to T-cells, instead, development of T-lymphocytes depends on thymic colonisation by blood borne progenitors (Le Douarin, Jotereau 1975). The earliest stages of T-cell development can be phenotypically distinguished by cell surface expression of CD44 and CD25. The earliest thymocyte subpopulation, the fraction containing the recent thymic immigrants, is known as the double negative 1 (DN1) population and is phenotypically heterogeneous (see Chapter 3), with the most potent T-cell differentiation potential residing in the fraction of cells expressing the receptor tyrosine kinase c-kit (CD117) (Matsuzaki, Gyotoku et al. 1993; Allman, Sambandam et al. 2003). Functionally, the DN1 CD117 positive progenitors (hereafter referred to as DN1 CD117) are multipotent at the population level, being able to generate B-cells, NK-cell and dendritic cells *in vivo* and myeloid progeny *in vitro* (Wu, Antica et al. 1991, Ardavin, Wu et al. 1993, Balciunaite, Ceredig et al. 2005).

Recently, a more extensive dissection of the DN1 population by CD117 and CD24 revealed multiple progenitor subsets with different functional capabilities to produce T- and non T-lineage cells (Porritt, Rumfelt et al. 2004). In addition, this study revealed an *in vitro* potential of the DN1 CD117 negative population to generate T-cells. The ability of the DN1 CD117 negative population to generate T-cells has been largely ignored, even though a mutation in the *c-kit* gene results in only a slight decrease in T-cell development (Rodewald, Kretzschmar et al. 1995) and further more, no effect on T-cell generation was observed once signalling through c-kit (CD117) was blocked (Matsuzaki, Gyotoku et al. 1993).

In this study, a population of DN1 CD117 negative progenitor thymocytes was identified expressing CD45R (B220) on the cell surface (hereafter referred to as DN1 CD45R). Previously, a bone marrow progenitor population bearing CD45R on the cell surface was characterised (Gounari, Aifantis et al. 2002). These cells, termed "common lymphoid progenitor 2" population, were isolated based on expression of human CD25 which was driven by the murine pre-Tα regulatory elements and in addition expressed CD127 (IL-7Rα) and low levels of CD117 on the cell surface. Functionally, this population could generate B- and T-cells in transplantation assays and was shown to be downstream of the common lymphoid progenitor population (Martin, Aifantis et al. 2003). High expression of pre-Tα at the genetic level and cell surface expression of CD45R aligns the common lymphoid progenitor-2 and the DN1 CD45R populations together, however, lack of CD117 and CD127 expression on the DN1 CD45R population illustrates a difference between these populations. Recently, a population of cells exhibiting an identical phenotype to the DN1 CD45R population was isolated from the blood of adult mice (Krueger, von Boehmer 2007). These cells had no capacity to generate any progeny upon transplantation into a *Rag2-/-cd132-/-* host. Lack of any differentiation potential was interpreted as an experimental limitation, since very few cells were isolatable (Krueger, von Boehmer 2007).

The DN1 CD45R population comprised a noticeable fraction of the total DN1 pool in C57BL/6 mice and a preponderant DN1 population amongst all other mouse strains analysed (see Chapter 3). Phenotypically, this population exhibited some cell surface expression of a T-cell specific marker CD90 and in addition ubiquitously expressed the EYFP reporter driven by the human CD2 Cre transgene, which so far has only been reported in the lymphoid lineage. The DN1 CD45R population also expressed genetic elements associated with active Notch signalling and T-cell differentiation, without any

evidence of B-cell genes (see Chapter 5). This could be an indication of a restricted, T-cell biased developmental potential, therefore this prompted a functional analysis of the DN1 CD45R population.

Results

Analysis of Progenitor Functional Potential *in vitro*

The ability of the DN1 CD45R population to generate mature lymphoid lineages was assessed. First, the ability to develop B-cell progeny was examined in a limiting dilution assay. Cells were plated into individual wells containing the OP9 bone marrow stromal cell layer, which has been shown to efficiently support B-cell development. Under these conditions the DN1 CD45R population did not generate any B-cell progeny, whereas the control population, the CD117+CD19+ pro-B-cell population isolated from the bone marrow, generated B-cell clones with an approximate frequency of 1 in 3 cells (Figure 6.1).

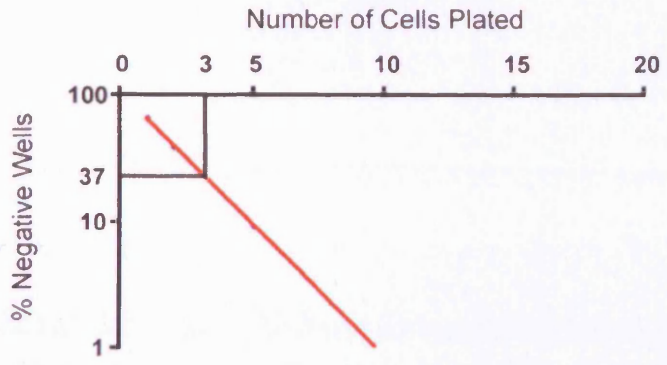
The DN1 CD45R population expressed genetic elements indicative of T-cell lineage developmental potential, namely Notch-1 and pre-Tα, therefore the ability to generate T-cell progeny was next examined by culturing purified cells on the OP9-Dll1 bone marrow stromal monolayer capable of supporting T-cell development. In parallel, the DN1 CD117 population was examined and served as a positive control, since these cells are the most prominent T-cell progenitors thus far identified in the thymus. Figure 6.2 illustrates the development of both progenitor populations along the well-defined pathway of T-cell differentiation. The DN1 CD45R population exhibited rapid kinetics of development. After 48 hours in culture, the DN1 CD45R population progressed to the DN4 stage of development without any amplification (Figures 6.2A and 6.3), whereas the DN1 CD117 population began the progression to the DN2 stage of development with noticeable expansion (Figures 6.2A and 6.3). After 72 hours of culture the DN1 CD45R population generated double positive cells, whereas the DN1 CD117 fraction progressed only to the DN2 stage of development (Figure 6.2B). After seven days in culture, whilst the DN1 CD117 population did not acquire CD4 or CD8,

the DN1 CD45R population generated TCR high single positive T-cells (Figure 6.2C). Throughout the time course of development, the DN1 CD117 fraction was dramatically amplified, whereas the DN1 CD45R population differentiated without any significant amplification (Figure 6.3), which is consistent with the quiescent state of the DN1 CD45R population (Figure 3.6B).

Figure 6.1 Assessment of B-cell Potential of the DN1 CD45R Population *in vitro*.

Purified progenitor cells from 4 weeks old female C57BL/6 mice were plated at 1, 2, 5, 10 and 20 cells per individual well on the OP9 bone marrow stromal monolayer, media supplemented with IL-7 (1 ng/ml), SCF (100 ng/ml) and Flt3L (5 ng/ml), and cultured for 14 days. The DN1 CD45R population did not give any B-cell clones, whereas the CD117+CD19+ pro-B-cell population isolated from bone marrow generated B-cell clones with an approximate frequency of 1 in 3 cells.

pro-B (CD117+CD19+)



DN1 CD45R

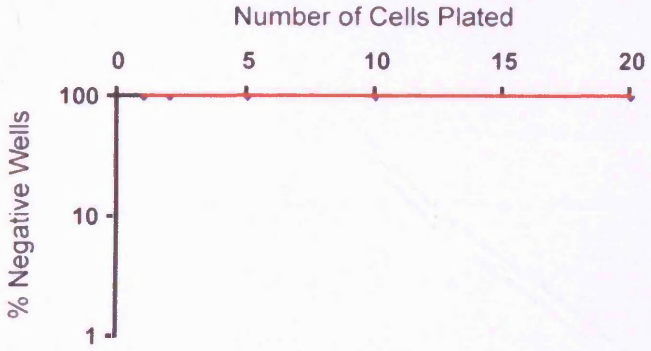


Figure 6.2 Assessment of T-cell Potential of the DN1 CD45R Population *in vitro*.

Purified progenitor populations from 4 weeks old female C57BL/6 mice were cultured on the OP9-Dll1 bone marrow stromal cell layer, capable of supporting T-cell development, media supplemented with IL-7 (1 ng/ml), SCF (100 ng/ml) and Flt3L (5 ng/ml). Representative histograms show the developmental progression of respective progenitor populations as assessed by cell surface expression of CD44, CD25, CD8 and CD4.

A Histograms representing the developmental progression of DN1 CD117 and DN1 CD45R progenitor thymocytes after 48 hours in culture. Some DN1 CD117 cells have progressed to the DN2 stage of development, whereas most DN1 CD45R cells have differentiated to the DN4 stage.

B Histograms representing the developmental progression of DN1 CD117 and DN1 CD45R progenitor thymocytes after 72 hours in culture. The DN1 CD117 population has progressed to the DN2 stage of development, whereas some DN1 CD45R cells have progressed to the double positive stage.

C Histograms representing the developmental progression of DN1 CD117 and DN1 CD45R progenitor thymocytes after 7 days in culture. The DN1 CD117 population did not upregulate CD4 or CD8, whereas the DN1 CD45R population generated some TCR positive single positive T-cells.

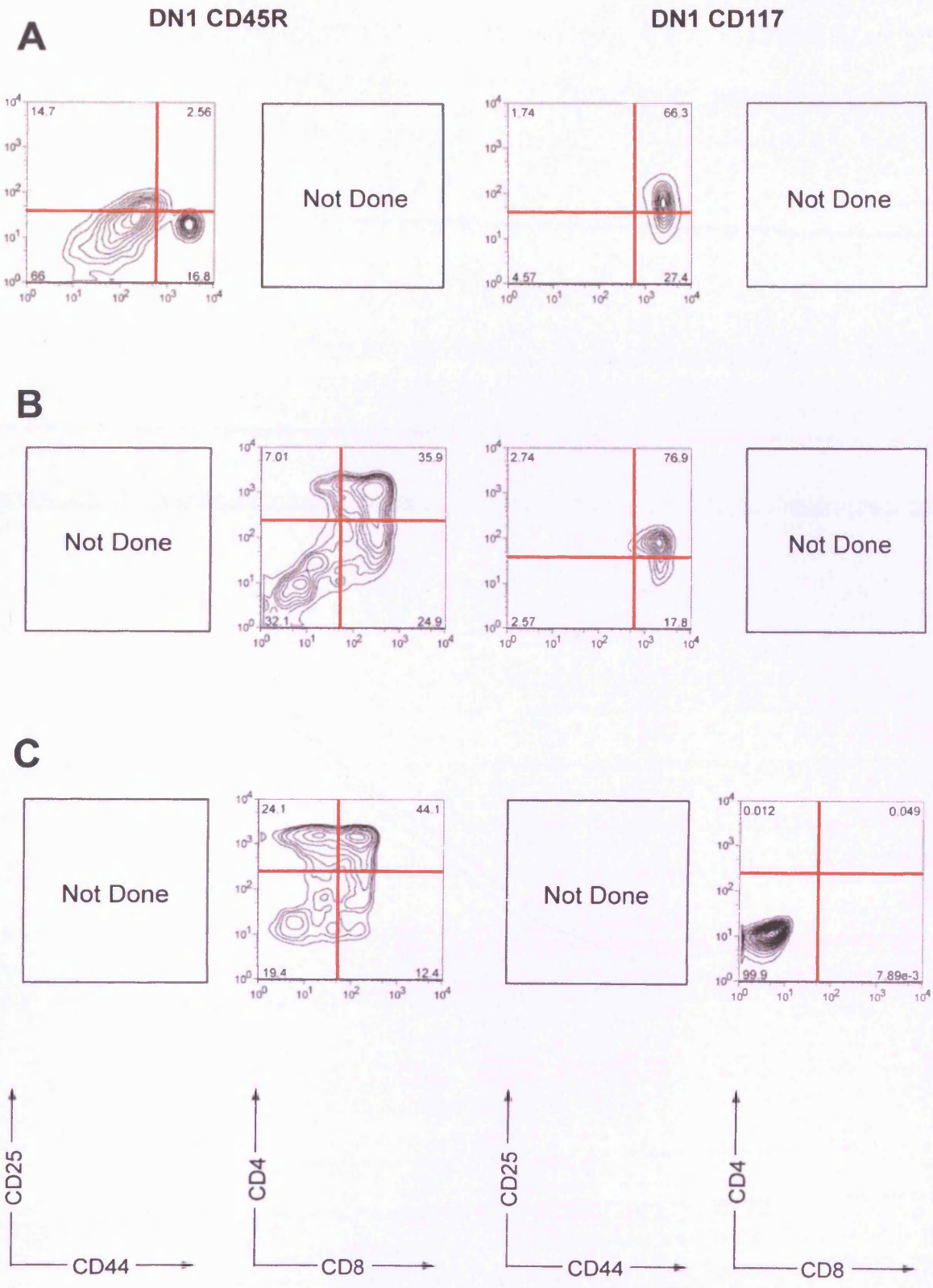
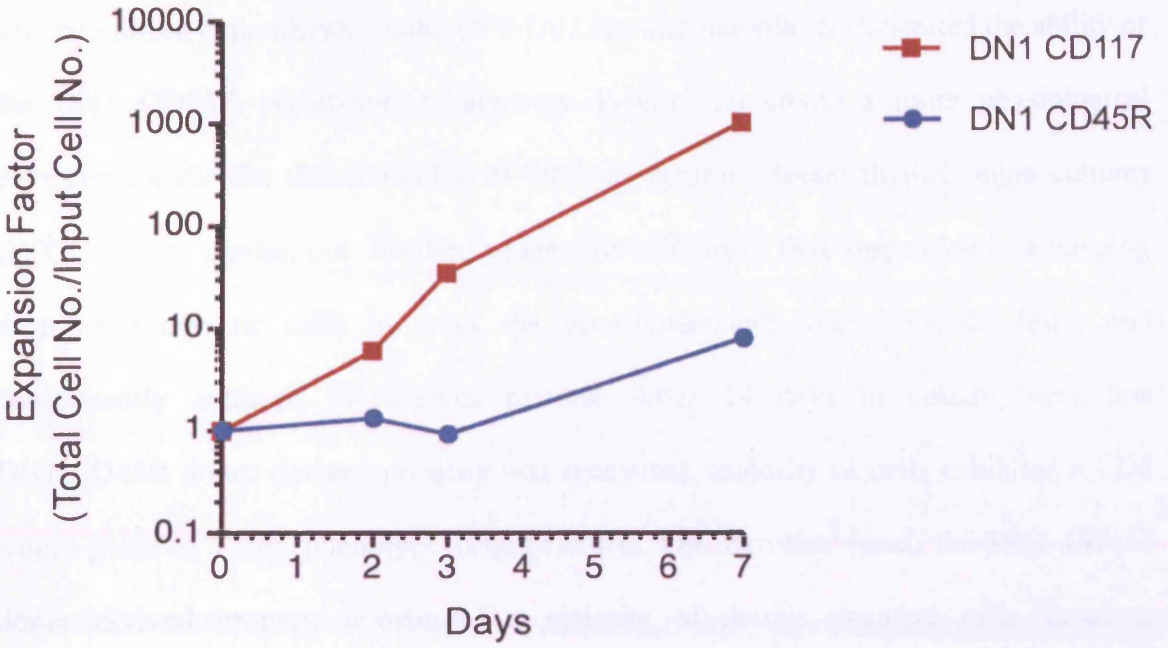

A
DN1 CD45R
DN1 CD117
14.7
2.56
66
16.8
Not Done
1.74
66.3
4.57
27.4
Not Done
B
Not Done
7.01
35.9
32.1
24.9
2.74
76.9
2.57
17.8
Not Done
C
Not Done
24.1
44.1
19.4
12.4
Not Done
0.012
0.049
99.9
7.89e-3
CD25
CD44
CD4
CD8
CD25
CD44
CD4
CD8

Figure 6.3 Proliferative Capacity of DN1 Progenitor Thymocytes *in vitro*.

The expansion factor of DN1 progenitors during the culture on OP9-Dll1 stromal monolayer. Cell numbers were counted at each indicated time point and expressed as a factor of the initial cellular input. The DN1 CD117 population was dramatically amplified, whereas the DN1 CD45R population showed only a minimal increase.


Expansion Factor
(Total Cell No./Input Cell No.)
10000
1000
100
10
1
0.1
0
1
2
3
4
5
6
7
Days
DN1 CD117
DN1 CD45R

The co-culture experiments on the OP9-Dll1 stromal monolayer illustrated the ability of the DN1 CD45R population to generate T-cells. To create a more physiological environment for the differentiation of DN1 progenitors, foetal thymic organ cultures (FTOCs) were carried out. Purified progenitor cells were first suspended in a hanging drop to allow the cells to enter the deoxyguanosine treated thymic lobes and subsequently cultured for various periods. After 14 days in culture, very few DN1 CD45R donor derived progeny was recovered, majority of cells exhibited a CD8 single positive T-cell phenotype (Figure 6.4A). On the other hand, the DN1 CD117 donor derived progeny constituted a majority of double negative cells, however immature CD8 single positive and double positive cells were generated (Figure 6.4A). After 21 days in FTOC, again, very few cells were recovered from the culture initiated with DN1 CD45R progenitor cells (Figure 6.4B), whereas a further developmental progression of DN1 CD117 donor cells was evident by a decrease in double negative cells and an increase in immature CD8 single positive and double positive cells (Figure 6.4B). This paralleled a significant expansion of DN1 CD117 cells during culture (Figure 6.4C).

Next, the developmental potential of thymocyte progenitors was tested at earlier time points. After 5 days of culture the DN1 CD45R population revealed a T-cell developmental potential illustrated by the generation of double positive cells (Figure 6.5A). Likewise, the DN1 CD117 population generated double positive cells even though majority remained at the double negative stage (Figure 6.5A). After 7 days, the donor derived double positive cells increased in both cultures (Figure 6.5B) and the DN1 CD117 population proliferated to a greater extent than the DN1 CD45R population (Figure 6.5D). At day 10 of culture a salient difference was observed between the donor derived progeny from the two DN1 populations. Whereas the DN1

CD45R progeny showed a profile comparable to day 7 of culture, the progeny of the DN1 CD117 population was predominantly composed of double negative cells (Figure 6.5C) and the loss of double positive cells was reflected in a decrease of cell recovery (Figure 6.5D).

In foetal thymic organ culture, the DN1 CD117 population also generated γ/δ T-cells, natural killer cells and dendritic cells (Figure 6.6A). No B-cell progeny was ever detected, however, once these cells were cultured on the OP9 bone marrow stromal cell monolayer, B-cell developmental potential was revealed (Figure 6.6B) illustrating the multipotent nature of the DN1 CD117 cells. The small numbers of progeny derived from the DN1 CD45R population precluded any further analysis of the multipotency of this population in FTOCs.

Figure 6.4 Assessment of T-cell Potential of the DN1 CD45R Population in Foetal Thymic Organ Culture I.

Purified CD45.1+ progenitor thymocytes from 4 weeks old female C57BL/6 mice were suspended in a hanging drop for 24 hours to allow the transmigration of cells into deoxyguanosine treated CD45.2 E14.5 foetal thymic lobes, then cultured for indicated period on floating filters in media without cytokines. Representative histograms illustrate the developmental progression of respective progenitor populations as assessed by CD4 and CD8 cell surface expression.

A Histograms representing the developmental progression of DN1 CD45R (left histogram) and DN1 CD117 (right histogram) progenitor thymocytes after 14 days in culture. The DN1 CD117 culture contained some immature CD8 single positive and double positive cells. Very few cells were recovered from the DN1 CD45R culture, most exhibiting a CD8 single positive phenotype.

B Histograms representing the developmental progression of DN1 CD45R (left histogram) and DN1 CD117 (right histogram) progenitor thymocytes after 21 days in culture. The DN1 CD117 culture contained increased frequencies of immature CD8 single positive and double positive cells. Again, very few cells were recovered from the DN1 CD45R culture.

C Expansion factor of DN1 CD45R (blue bars) and DN1 CD117 (maroon bars) progenitor thymocytes after FTOC. The DN1 CD117 population was dramatically amplified.

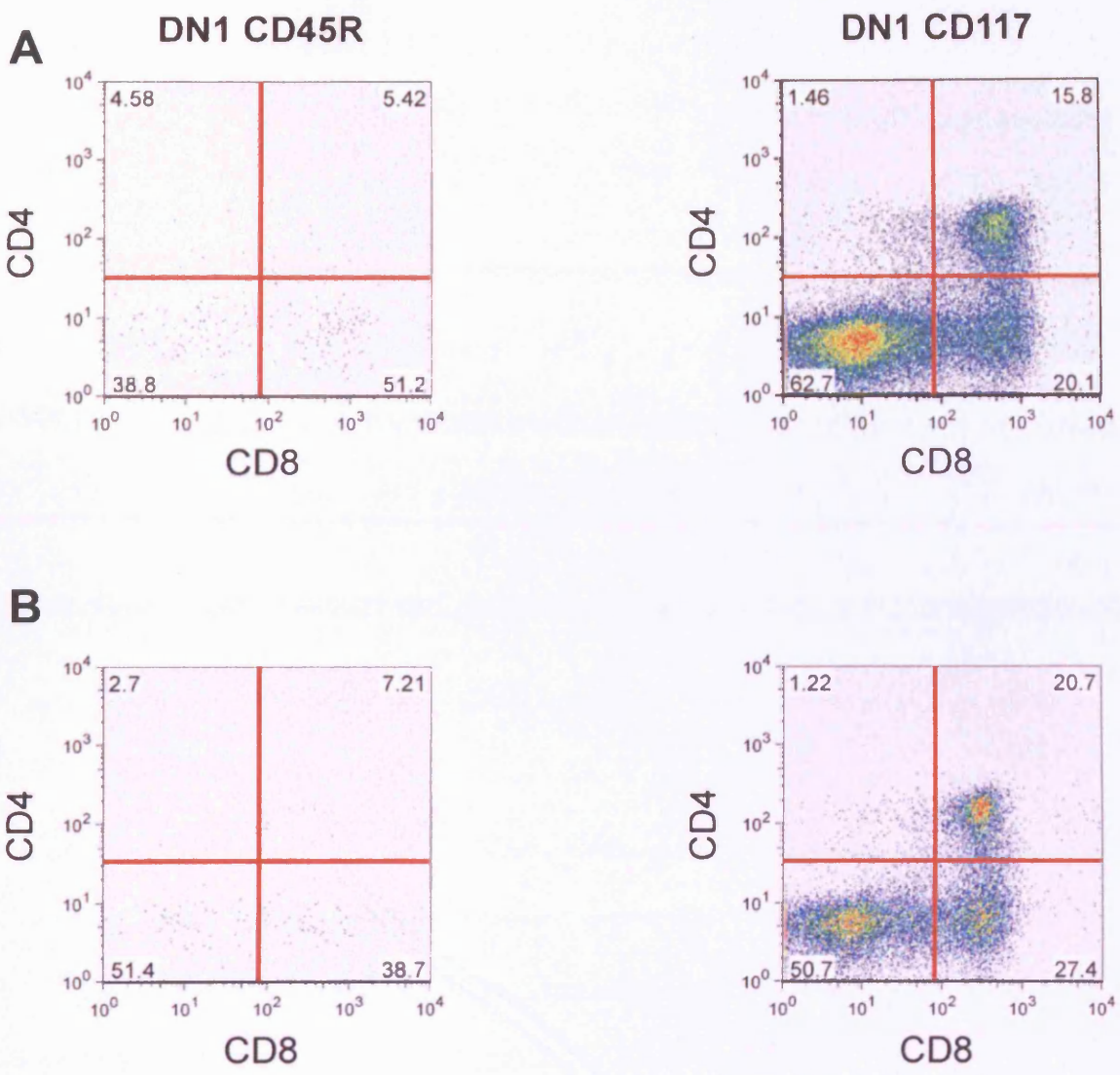

A
DN1 CD45R
DN1 CD117
CD4
CD8
4.58
5.42
38.8
51.2
1.46
15.8
62.7
20.1
B
2.7
7.21
51.4
38.7
1.22
20.7
50.7
27.4
C
Expansion Factor
(Total Cell No./Input Cell No.)
10
1
0.1
0.01
Day 14
Day 21
DN1 CD117
DN1 CD45R

Figure 6.5 Assessment of T-cell Potential of the DN1 CD45R Population in Foetal Thymic Organ Culture II.

Purified CD45.1+ progenitor thymocytes from 4 weeks old female C57BL/6 mice were suspended in a hanging drop for 24 hours to allow the transmigration of cells into deoxyguanosine treated CD45.2 E14.5 foetal thymic lobes, then cultured for indicated period on floating filters in media without cytokines. Representative histograms illustrate the developmental progression of respective progenitor populations as assessed by CD4 and CD8 cell surface expression.

A Histograms representing the developmental progression of DN1 CD45R (left histogram) and DN1 CD117 (right histogram) progenitor thymocytes after 5 days in culture. Some double positive cells are evident in both cultures.

B Histograms representing the developmental progression of DN1 CD45R (left histogram) and DN1 CD117 (right histogram) progenitor thymocytes after 7 days in culture. There is an increase of double positive cells in both cultures.

C Histograms representing the developmental progression of DN1 CD45R (left histogram) and DN1 CD117 (right histogram) progenitor thymocytes after 10 days in culture. Most DN1 CD117 progeny do not express CD4 or CD8, whereas some DN1 CD45R cells have progressed to the CD4 or CD8 single positive stage.

D Expansion factor of DN1 CD45R (blue bars) and DN1 CD117 (maroon bars) progenitor thymocytes after FTOC. Both fractions exhibited a peak of expansion after 7 days in this experiment.

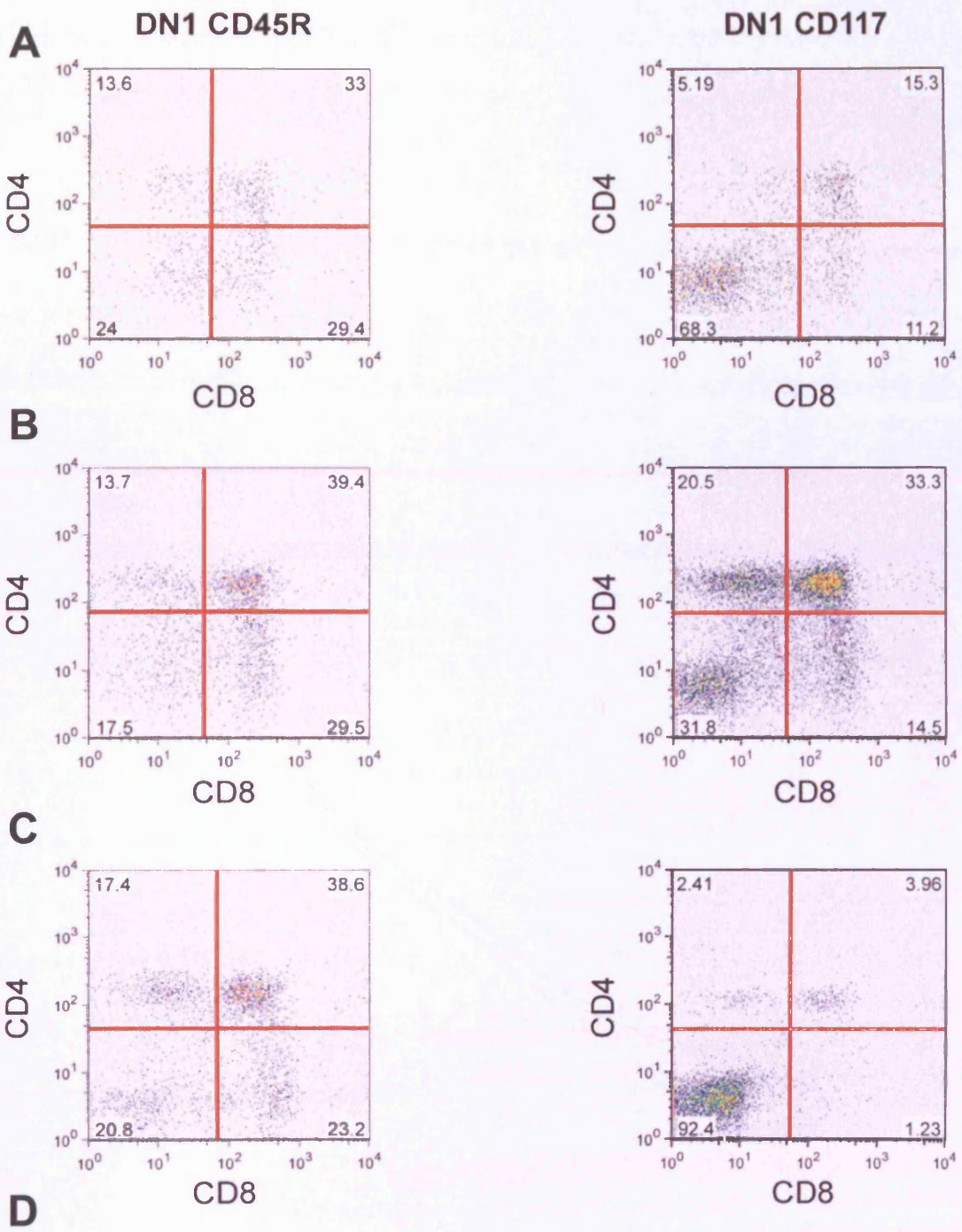
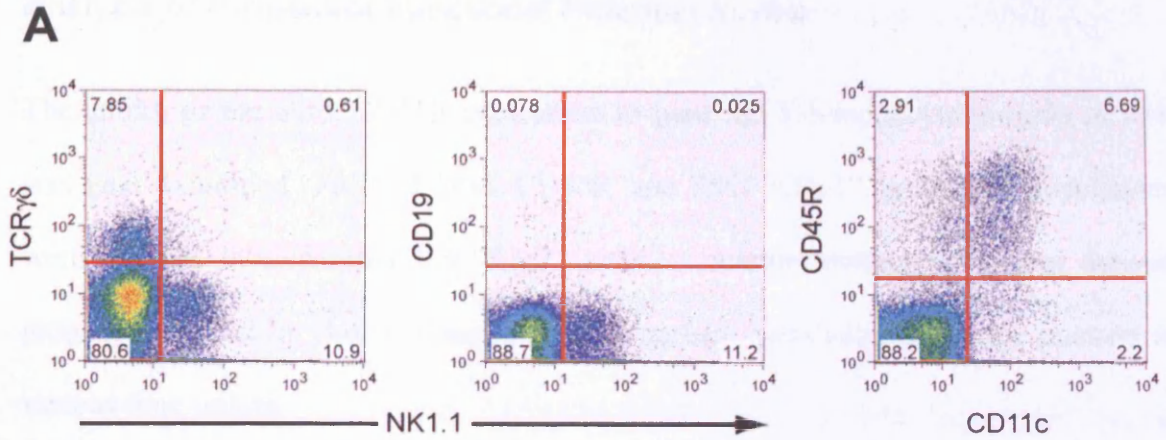

A
DN1 CD45R
DN1 CD117
13.6
33
24
29.4
5.19
15.3
68.3
11.2
B
13.7
39.4
17.5
29.5
20.5
33.3
31.8
14.5
C
17.4
38.6
20.8
23.2
2.41
3.96
92.4
1.23
CD4
CD8
D
Expansion Factor
(Total Cell No./Input Cell No.)
10
1
0.1
0.01
Day 0
Day 5
Day 7
Day 10
DN1 CD117
DN1 CD45R

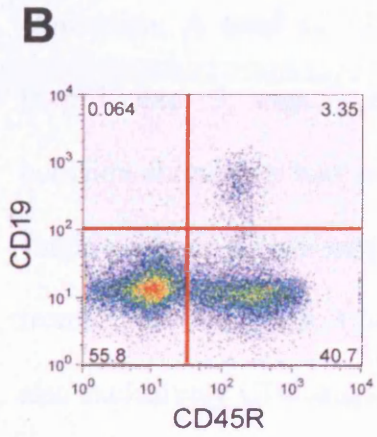
Figure 6.6 Analysis of Functional Potential of the DN1 CD117 Population *in vitro*.

A Histograms representing the capacity of DN1 CD117 cells from 4 weeks old C57BL/6 female mice to generate alternative haematopoietic lineages after 14 days in foetal thymic organ culture, as assessed by cell surface expression of lineage markers. The DN1 CD117 cells could generate γ/δ T-cells, natural killer cells, and dendritic cells under the FTOC conditions.

TCRγδ+ – γ/δ T-cells, NK1.1+ – natural killer cells, CD11c/+CD45R+ – dendritic cells.

B Histograms representing the ability of the DN1 CD117 cells from 4 weeks old C57BL/6 female mice to generate B-cell progeny on the OP9 bone marrow stromal cell monolayer after 14 days in culture, media supplemented with IL-7 (1 ng/ml), SCF (100 ng/ml) and Flt3L (5 ng/ml), as assessed by cell surface expression of CD19 and CD45R.


A
TCRγδ
7.85
0.61
80.6
10.9
CD19
0.078
0.025
88.7
11.2
CD45R
2.91
6.69
88.2
2.2
NK1.1
CD11c


B
CD19
0.064
3.35
55.8
40.7
CD45R

Analysis of Progenitor Functional Potential *in vivo*

The ability of the DN1 CD45R population to generate T-lymphocyte progeny *in vivo* was next examined. Purified DN1 CD45R and DN1 CD117 progenitor populations were injected intravenously into *Rag2-/-cd132-/-* double mutants and donor derived progeny analysed by flow cytometry for cell surface expression of lineage markers at various time points.

At 4 weeks, mice reconstituted with the DN1 CD45R population showed rare chimerism. A total of 12 mice were analysed from 3 separate sorting experiments [exp. 1, exp. 2, exp. 3] and overall only 4 mice exhibited reconstitution. On one occasion chimerism was seen in the thymus. These donor derived cells constituted CD8 single positive T-cells only (Figure 6.7A and Table 6.1). At 5 weeks (2 chimeric mice from 2 analysed mice, 1 experiment [exp. 4]), donor derived cells in the thymus were also exclusively CD8 single positive T-cells (Figure 6.7A and Table 6.1). At 6 weeks no donor-derived cells were evident in the thymus of hosts transplanted with DN1 CD45R progenitor thymocytes (2 chimeric mice from 4 analysed mice, 2 separate experiments [exp. 4 and exp. 5]).

On the other hand, the DN1 CD117 population showed good reconstituting ability. At 4 weeks, chimerism was observed in the thymus of 11 mice from a total of 15 analysed animals [exp. 1, exp.2, exp. 3]. At this stage, majority of donor-derived cells were in the double negative compartment (Table 6.1), however, some hosts contained more mature donor-derived thymocytes. By 5 weeks the DN1 CD117 chimeric mice (2 chimeric from 2 analysed, 1 experiment [exp. 4]) showed an increase in proportion of double as well as single positive thymocytes (Figure 6.7B and Table 6.10), therefore illustrating the developmental progression of DN1 CD117 progenitor cells through the well-defined stages of T-cell development. T-cell differentiation of DN1 CD117 cells was completed

by six weeks, which is indicated by the absence of double negative as well as double positive thymocytes (see Table 6.10) (3 chimeric mice from 5 analysed mice, 2 experiments [exp.4 and exp. 5]).

Excluding T-cells, all other blood lineages develop in the bone marrow of an adult organism, therefore this organ was analysed for the presence of donor-derived progeny. At 4 weeks, the bone marrow of animals reconstituted with the DN1 CD45R population illustrated a bias towards the CD8NKT-cell lineage, with very rare CD4 T-cells being detected (Figure 6.8A and B, Table 6.1). There was no evidence of any B- or myeloid cell generation from the DN1 CD45R population. A similar phenotype was observed 5 and 6 weeks after transplantation with the DN1 CD45R population, nearly all cells expressed CD8 and NK1.1 (Table 6.1). On one occasion, [exp. 5], a significant population of γ/δ T-cells was detected after 6 weeks. The bone marrow of the DN1 CD117 chimeras illustrated multilineage reconstitution, mirrored in the presence of T-, B-, NK- and even very rare myeloid cells (Figure 6.8C and D, Table 6.1). At 4 weeks, the preponderant lineage was the NK lineage, with the proportion of this cell type declining through time. B-cell development was detected at 4 weeks, however by 5 weeks, all progenitor B-cells subsequently migrated out of the bone marrow to complete maturation in the spleen. The peak of myeloid development was observed after five weeks post transplantation (Table 6.1).

Leukocytes navigate the body via the blood. A good representation of the peripheral white blood cell composition is the spleen, thus this organ was analysed for the presence of donor-derived cells. The progeny of the DN1 CD45R population were CD8 T-cells, with varying levels of expression of the NK1.1 molecule (Figure 6.9A and B). Once again, CD4 T-cells were detected with very low frequency only after 4 weeks. This illustrates that the DN1 CD45R population has a very limited potential to generate

differentiated progeny *in vivo*. In contrast, the progeny of the DN1 CD117 population was represented in all blood cell lineages, underlining the multipotent nature of this population (Figure 6.9C and D, Table 6.1).

Since the DN1 CD45R population developed with rapid kinetics *in vitro*, and the classical stages of thymic T-cell development were not detected after transplantation, an earlier time point was analysed to check the developmental progression of this population. To circumvent the possibility of contamination, 1 x 10^3 cells were injected into the tail vein of *Rag2-/-cd132-/-* double mutants and donor derived progeny analysed after 2 and 4 weeks post transplantation. At 2 weeks, no donor-derived progeny was detected in 8 animals injected with the DN1 CD45R population on 2 separate occasions [exp. 6 and exp 7], while evidence of T-cell development was prominent in the thymi of the DN1 CD117 chimeras (Table 6.2) (4 reconstituted mice from 8 injected mice [exp. 6 and exp. 7]). There was clear B-cell and a dominant process of NK-cell development taking place in the bone marrow of the DN1 CD117 chimeras (Table 6.2). The periphery of hosts transplanted with the DN1 CD117 population contained mainly NK-cells. At 4 weeks, the DN1 CD45R population generated CD8NKT-cells on one occasion only, (8 injected mice, 2 independent experiments), which corresponds to previous *in vivo* experiments, once again exhibiting a very limited functional potential of these cells. In contrast, the DN1 CD117 population displayed the potential to generate T-, B-, NK- and some myeloid cell offspring after 4 weeks (Table 6.2), thus illustrating the multipotent nature of this progenitor fraction at the population level.

Figure 6.7 Developmental Potential of Thymic Progenitors *in vivo* – Thymus.

Representative histograms depicting the developmental progression of 5 x 10^3 EYFP+ DN1 CD45R or 5 x 10^3 EYFP+ DN1 CD117 purified thymic progenitor cells from 4 weeks old female C57BL/6 mice after intravenous administration into sub-lethally irradiated 6-8 week old female *Rag2-/-cd132-/-* hosts. Thymi of recipient mice were mechanically disrupted to prepare single cell thymocyte suspensions, which were subsequently stained for CD4, CD8 and T-cell receptor.

A Representative histograms illustrating the developmental progression of the DN1 CD45R progenitor population in the thymus of recipient animals as assessed by cell surface expression of CD4, CD8 and T-cell receptor (TCR) on donor derived EYFP+ cells. Only donor derived CD8 single positive T-cells were detected in the thymus.

B Representative histograms illustrating the developmental progression of the DN1 CD117 progenitor population in the thymus of recipient animals as assessed by cell surface expression of CD4, CD8 and T-cell receptor (TCR) on donor derived EYFP positive cells. The DN1 CD117 progenitor population progressed through well-defined stages of T-cell development as illustrated by the presence of double positive and CD8 or CD4 single positive T-cells.

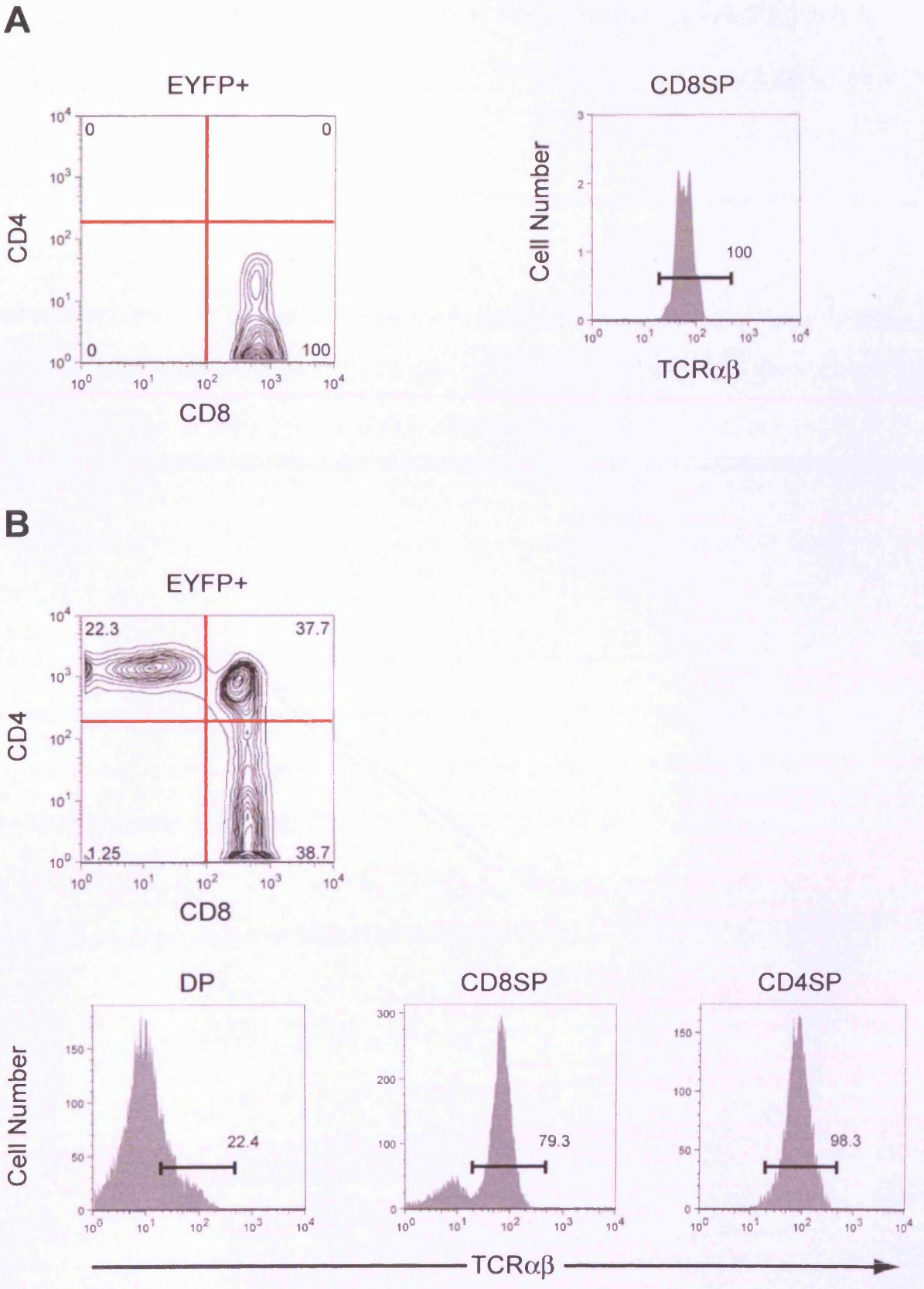

A
EYFP+
CD4
CD8
0
0
0
100
CD8SP
Cell Number
100
TCRαβ
B
EYFP+
CD4
CD8
22.3
37.7
1.25
38.7
DP
CD8SP
CD4SP
Cell Number
22.4
79.3
98.3
TCRαβ

Figure 6.8 Developmental Potential of Thymic Progenitors *in vivo* – Bone Marrow.

Representative histograms depicting the developmental progression of 5 x 10^3 EYFP+ DN1 CD45R or 5 x 10^3 EYFP+ DN1 CD117 purified thymic progenitor cells from 4 weeks old female C57BL/6 mice after intravenous administration into sub-lethally irradiated 6-8 week old female *Rag2-/-cd132-/-* hosts. Femoral long bones of recipient mice were flushed to prepare single cell bone marrow suspensions, which were subsequently stained for lineage specific cell surface markers. Cell surface expression of CD4 or CD8 concomitantly with T-cell receptor marks mature T-cells, expression of γ/δ T-cell receptor marks γ/δ T-cells, expression of CD19 and CD45R marks B-cells and B-cell progenitors, cell surface expression of NK1.1 identifies natural killer cells, and expression of CD11b together with Ly6G phenotypically defines macrophages.

A Representative histograms illustrating the DN1 CD45R derived progeny in the bone marrow of recipient mice. Majority of cells were CD8 single positive T-cells.

B Representative histogram illustrating the DN1 CD45R progeny all expressed the natural killer cell marker NK1.1.

C Representative histograms illustrating the DN1 CD117 derived progeny in the bone marrow of recipient mice. Donor derived CD4 and CD8 α/β T-cells were present in the bone marrow.

D Representative histograms illustrating the DN1 CD117 derived progeny in the bone marrow constituted γ/δ T-cells, B-cells, natural killer cells and some myeloid cells.

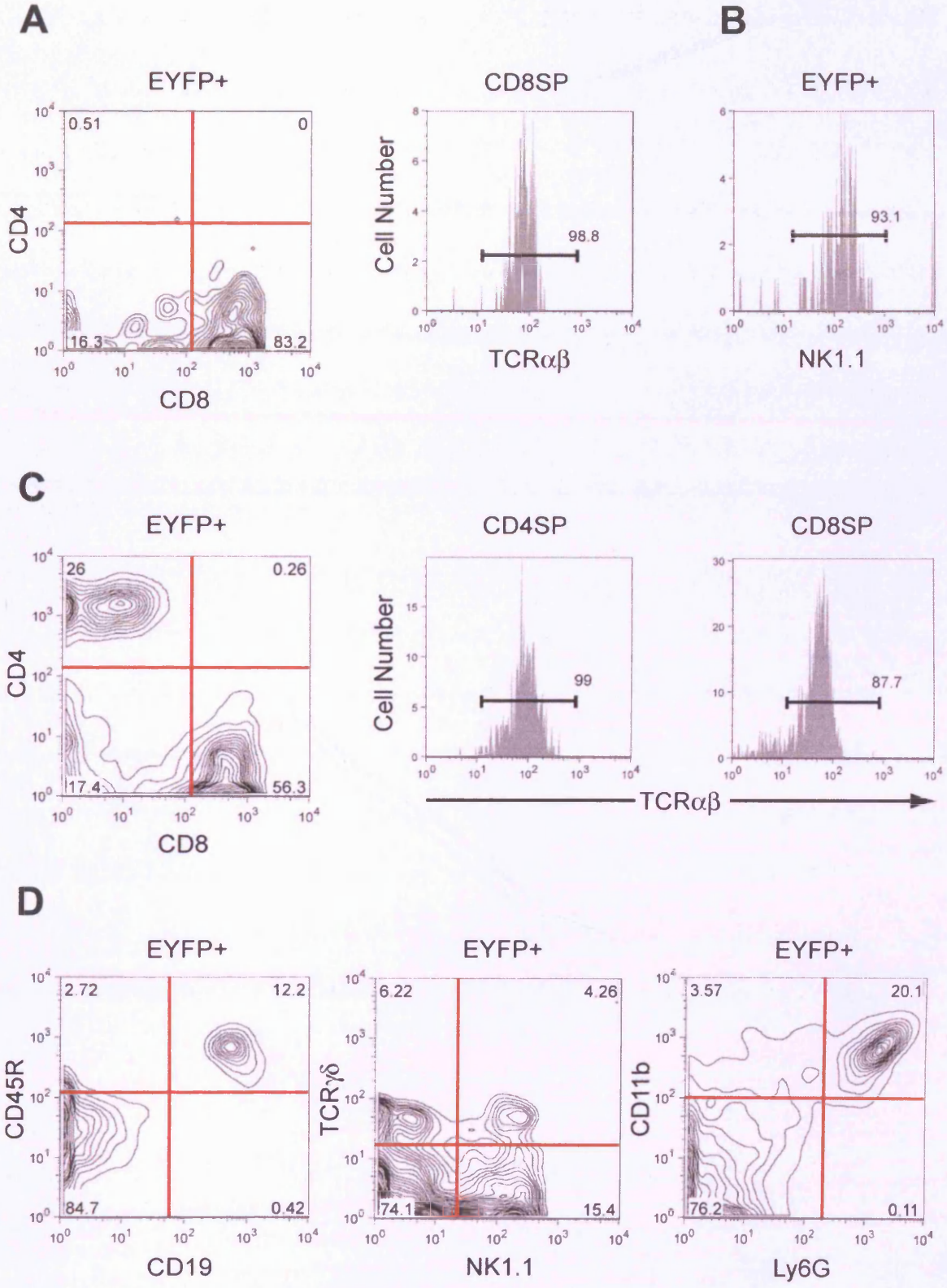

A
EYFP+
0.51
0
16.3
83.2
CD4
CD8
CD8SP
98.8
Cell Number
TCRαβ
B
EYFP+
93.1
NK1.1
C
EYFP+
26
0.26
17.4
56.3
CD4
CD8
CD4SP
99
Cell Number
CD8SP
87.7
TCRαβ
D
EYFP+
2.72
12.2
84.7
0.42
CD45R
CD19
EYFP+
6.22
4.26
74.1
15.4
TCRγδ
NK1.1
EYFP+
3.57
20.1
76.2
0.11
CD11b
Ly6G

Figure 6.9 Developmental Potential of Thymic Progenitors *in vivo* – Spleen.

Representative histograms depicting the developmental progression of 5 x 10^3 EYFP+ DN1 CD45R or 5 x 10^3 EYFP+ DN1 CD117 purified thymic progenitor cells from 4 weeks old female C57BL/6 mice after intravenous administration into sub-lethally irradiated 6-8 weeks old female *Rag2-/-cd132-/-* hosts. Spleens of recipient mice were mechanically disrupted to prepare single cell suspensions, which were subsequently stained for lineage specific cell surface markers. Cell surface expression of CD4 or CD8 concomitantly with T-cell receptor marks mature T-cells, expression of γ/δ T-cell receptor marks γ/δ T-cells, expression of CD19 and CD45R marks B-cells and B-cell progenitors, cell surface expression of NK1.1 identifies natural killer cells, and expression of CD11b together with Ly6G phenotypically defines macrophages.

A Representative histograms illustrating the DN1 CD45R derived progeny in the spleen of recipient mice. Virtually all cells were CD8 single positive T-cells.

B Representative histogram illustrating the expression of the natural killer cell marker, NK1.1, on the DN1 CD45R progeny. A few cells expressed this marker.

C Representative histograms illustrating the DN1 CD117 derived progeny in the spleen of recipient mice. Donor derived CD4 and CD8 α/β T-cells were present in the spleen.

D Representative histograms illustrating the DN1 CD117 derived progeny in the spleen constituted γ/δ T-cells, B-cells, natural killer cells and some myeloid cells.

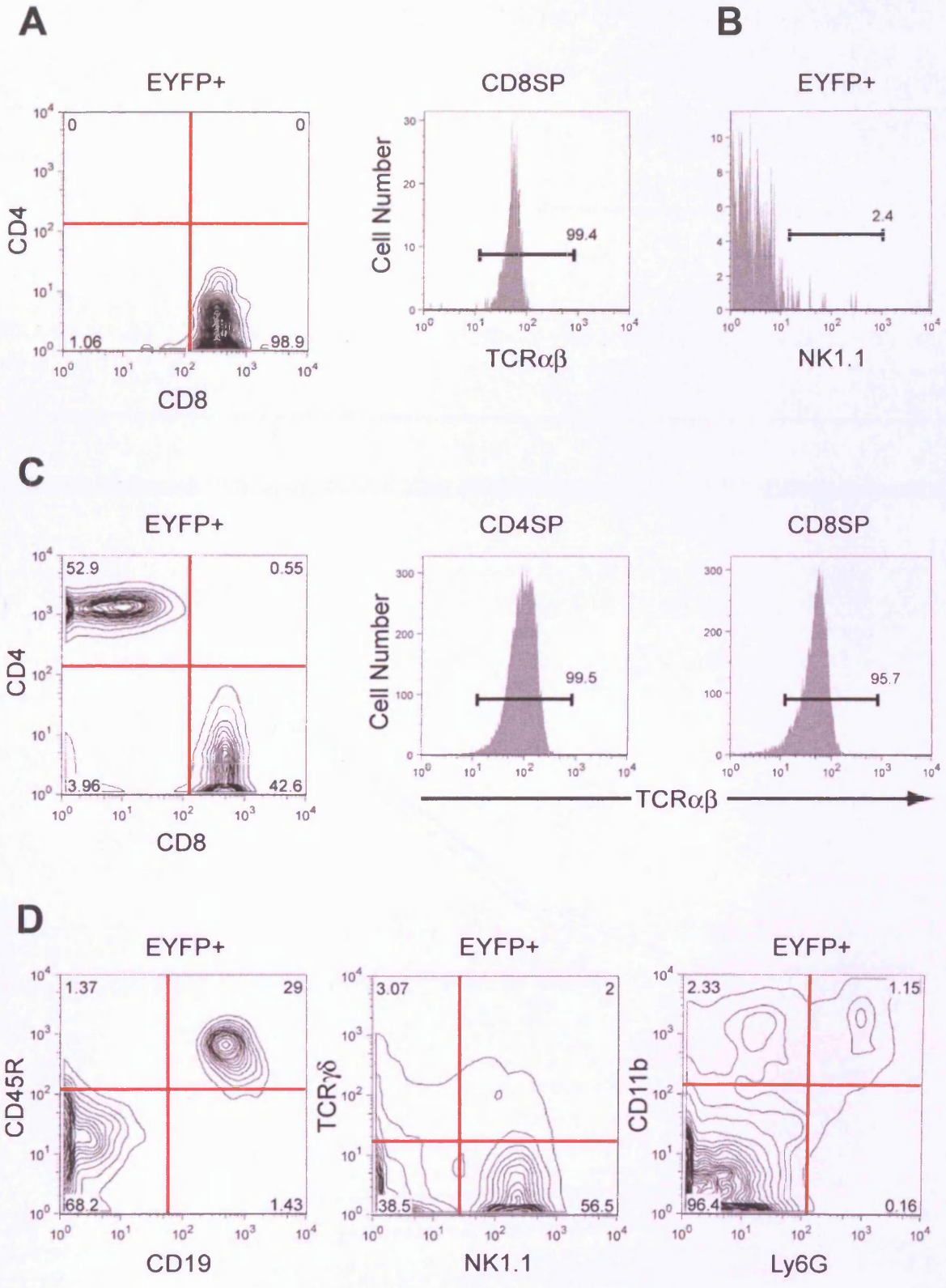

A
EYFP+
0
0
1.06
98.9
CD4
CD8
CD8SP
99.4
Cell Number
TCRαβ
B
EYFP+
2.4
NK1.1
C
EYFP+
52.9
0.55
3.96
42.6
CD4
CD8
CD4SP
99.5
CD8SP
95.7
Cell Number
TCRαβ
D
EYFP+
1.37
29
68.2
1.43
CD45R
CD19
EYFP+
3.07
2
38.5
56.5
TCRγδ
NK1.1
EYFP+
2.33
1.15
96.4
0.16
CD11b
Ly6G

Table 6.1 Developmental Potential of DN1 Thymic Progenitors *in vivo* I.

5 x 10^3 EYFP+ DN1 CD45R or 5 x 10^3 EYFP+ DN1 CD117 purified thymic progenitor cells from 4 weeks old female C57BL/6 mice were intravenously injected into the tail of sub-lethally irradiated (250 rads) 6-8 weeks old female *Rag2-/-cd132-/-* recipients. Donor derived progeny was analysed by staining cells from thymus, bone marrow and spleen preparations with lineage specific surface markers and analysing by flow cytometry at indicated time points, gating on EYFP+ cells. Values represent averaged frequency ± standard deviation of donor-derived EYFP+ progeny in reconstituted mice (n) only. The 4 weeks time point amalgamates 3 independent experiments [exp. 1, exp. 2 and exp. 3], the 5 weeks time point comprises one experiment [exp. 4] and the 6 weeks time point constitutes 2 independent experiments [exp. 4 and exp 5].

DN – CD4/CD8 double negative cells, ISP – CD8 positive/TCR negative immature single positive cells, DP – CD4 positive/CD8 positive cells, CD4 – CD4 positive/TCR positive T-cells, CD8 – CD8 positive/TCRβ positive cells, γδ – γ/δ T-cells, B – CD19 positive/CD45R positive B-cells, NK – NK1.1 positive natural killer cells, myeloid – CD11b positive/Ly6G positive myeloid cells.

	4 Weeks		5 Weeks		6 Weeks	
	DN1 CD45R (n=4)	DN1 CD117 (n=11)	DN1 CD45R (n=2)	DN1 CD117 (n=2)	DN1 CD45R (n=2)	DN1 CD117 (n=3)
Thymus						
	Av. +/- S.D. (%)	Av. +/- S.D. (%)	Av. +/- S.D. (%)	Av. +/- S.D. (%)	Av. +/- S.D. (%)	Av. +/- S.D. (%
DN	0.00	51.1 +/- 41.6	0.00	22.9 +/- 21.7	0.00	0.2 +/- 0.3
ISP	0.00	2.7 +/- 4.5	0.00	8.1 +/- 2.7	0.00	0.00
DP	0.00	9.0 +/- 21.3	0.00	20.9 +/- 16.8	0.00	0.4 +/- 0.6
CD4	0.00	14.9 +/- 18.0	0.00	11.2 +/- 11.2	0.00	23.3 +/- 33.0
CD8	25 +/- 50	21.1 +/- 24.3	99.3 +/- 0.7	37.0 +/- 9.1	0.00	9.4 +/- 13.3
BM						
CD4	6.8 +/- 11.8	16.5 +/- 17.3	0.00	13.9 +/- 11.8	0.00	12.0 +/- 14.4
CD8	35.5 +/- 40.5	29.1 +/- 27.1	87.4 +/- 5.8	43.9 +/- 6.7	95.9 +/- 2.7	39.4 +/- 27.9
γδ	0.00	8.2 +/- 8.2	1.4 +/- 1.4	5.4 +/- 5.2	26.1 +/- 23.0	19.7 +/- 20.9
B	0.00	2.0 +/- 3.6	0.00	0.00	0.00	2.2 +/- 3.1
NK	62.9 +/- 38.8	51.5 +/- 28.6	60.6 +/- 31.9	39.2 +/- 24.0	66.6 +/- 33.5	39.9 +/- 23.3
Myeloid	0.00	0.4 +/- 0.3	0.00	7.1 +/- 5.4	0.00	0.7 +/- 1.0
Spleen						
CD4	20.3 +/- 15.8	21.4 +/- 24.8	0.00	26.8 +/- 25.8	0.00	19.6 +/- 25.0
CD8	42.2 +/- 29.5	20.5 +/- 20.1	91.4 +/- 7.2	51.1 +/- 10.5	96.3 +/- 3.7	15.8 +/- 16.9
γδ	0.00	6.0 +/- 6.6	8.2 +/- 8.2	1.4 +/- 0.02	2.7 +/- 2.7	8.1 +/- 6.4
B	0.00	11.0 +/- 13.8	0.00	0.8 +/- 0.8	0.00	24.7 +/- 34.9
NK	51.6 +/- 30.7	40.6 +/- 31.8	23.3 +/- 21.7	16.6 +/- 15.4	53.2 +/- 46.8	29.6 +/- 33.8
Myeloid	0.00	0.04 +/- 0.05	0.00	1.9 +/- 1.7	0.00	1.1 +/- 0.8

Figure 6.10 Proliferative Capacity of DN1 Progenitor Thymocytes *in vivo*.

The expansion factor of DN1 progenitors *in vivo*. Total cell numbers from thymus, bone marrow and spleen were counted at each indicated time point and expressed as a factor of the initial cellular input. The DN1 CD117 population was dramatically amplified, whereas the DN1 CD45R population showed no amplification.

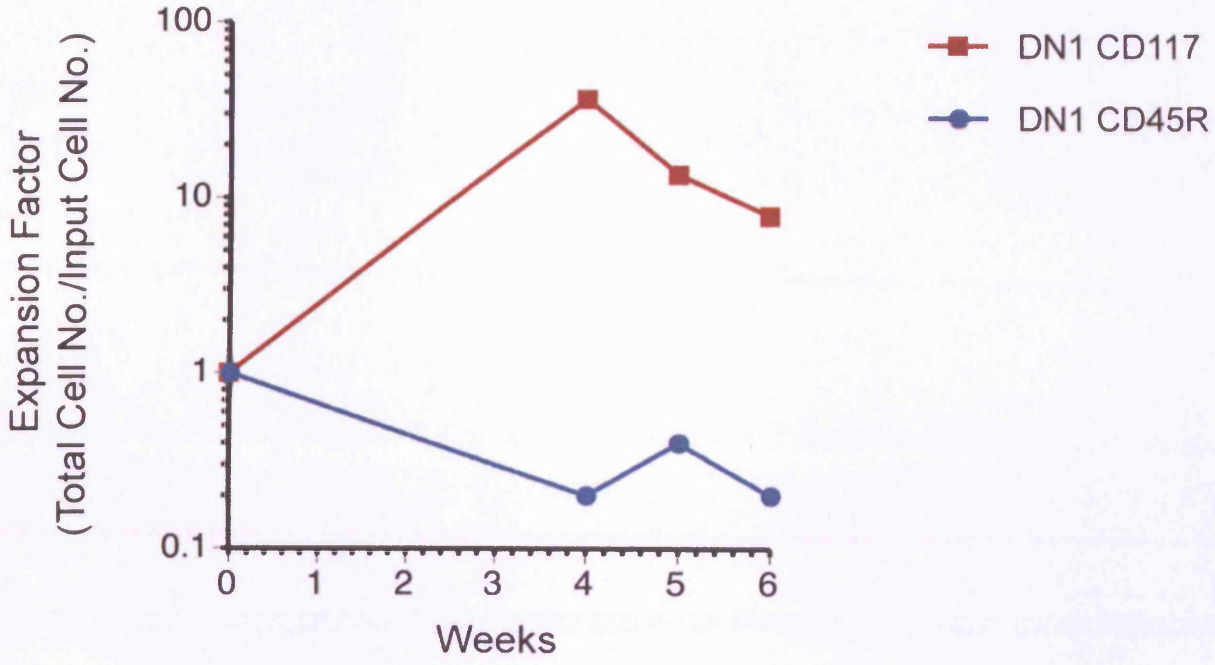

Expansion Factor
(Total Cell No./Input Cell No.)
100
10
1
0.1
0
1
2
3
4
5
6
Weeks
DN1 CD117
DN1 CD45R

Table 6.2 Developmental Potential of DN1 Thymic Progenitors *in vivo* II.

1 x 10^3 EYFP+ DN1 CD45R or 1 x 10^3 EYFP+ DN1 CD117 purified thymic progenitor cells from 4 weeks old female C57BL/6 mice were intravenously injected into the tail of sub-lethally irradiated (250 rads) 6-8 weeks old female *Rag2-/-cd132-/-* recipients. Donor derived progeny was analysed by staining cells from thymus, bone marrow and spleen preparations with lineage specific surface markers and analysing by flow cytometry at indicated time points, gating on EYFP+ cells. Values represent averaged frequency of donor derived EYFP+ cells ± standard deviation in reconstituted mice (n) only from 2 independent experiments [exp. 6 and exp. 7].

DN – CD4/CD8 double negative cells, ISP - CD8 positive/TCR negative immature single positive cells, DP – CD4 positive/CD8 positive cells, CD4 – CD4 positive/TCR positive T-cells, CD8 – CD8 positive/TCRβ positive cells, γδ – γδ T-cells, B – CD19 positive/CD45R positive B-cells, NK – NK1.1 positive natural killer cells, myeloid – CD11b positive/Ly6G positive myeloid cells.

	2 Weeks		4 Weeks	
	DN1 CD45R (n=0)	DN1 CD117 (n=4)	DN1 CD45R (n=1)	DN1 CD117 (n=6)
Thymus				
	Av. +/- S.D. (%)	Av. +/- S.D. (%)	Av. +/- S.D. (%)	Av. +/- S.D. (%)
DN	0.00	50.5 +/- 15.4	0.00	14.3 +/- 28.3
ISP	0.00	11.5 +/- 8.7	0.00	16.3 +/- 32.0
DP	0.00	40.6 +/- 24.1	0.00	2.8 +/- 4.7
CD4	0.00	0.00	0.00	0.00
CD8	0.00	0.00	0.00	0.00
BM				
CD4	0.00	0.2 +/- 0.4	0.00	4.3 +/- 8.3
CD8	0.00	0.00	87.80	2.7 +/- 2.9
γδ	0.00	3.1 +/- 3.2	0.00	56.3 +/- 33.3
B	0.00	6.0 +/- 10.4	0.00	3.9 +/- 5.5
NK	0.00	84.4 +/- 14.3	86.00	26.5 +/- 20.2
Myeloid	0.00	0.00	0.00	0.8 +/- 1.8
Spleen				
CD4	0.00	0.00	0.00	1.5 +/- 1.6
CD8	0.00	0.00	100.00	1.5 +/- 2.2
γδ	0.00	2.4 +/- 3.1	0.00	30.7 +/- 21.6
B	0.00	0.6 +/- 1.1	0.00	10.2 +/- 22.9
NK	0.00	79.7 +/- 11.6	70.00	48.9 +/- 23.4
Myeloid	0.00	0.00	0.00	0.4 +/- 0.7

Analysis of Progenitor Functional Potential in Athymic Mice

The DN1 CD45R population did not show any evidence of generating double positive cells in the thymus and additionally, the phenotype of DN1 CD45R donor derived cells was similar to cells which develop extrathymically, such as the CD8+ intraepithelial lymphocytes (IEL), therefore the dependence of the DN1 CD45R population on the thymus was addressed.

Purified progenitor populations were intravenously injected into *nu/nu* recipients which do not have a functional thymus, therefore any development of T-cells would take place extrathymically.

A total of 8 injected mice from 1 experiment did not show any evidence of donor derived T-cells from the DN1 CD45R population, whereas the DN1 CD117 progenitor cells generated progeny with a CD4-CD8-CD3ε+ phenotype (Table 6.3). These cells did not express CD4 or CD8 on the cell surface and therefore were not conventional α/β T-cells. However, expression of CD90.1 and CD3ε would identify these cells as γ/δ T-cells. This experiment illustrates the ability of the DN1 CD117 population to develop extrathymically, whereas the DN1 CD45R progenitor population requires a functional thymus for development.

Table 6.3 Developmental Potential of DN1 Thymic Progenitors in an Athymic Environment.

5 x 10^3 CD90.1 DN1 CD45R or 5 x 10^3 CD90.1 DN1 CD117 purified progenitor thymocytes from 6-10 week old BALB/c mice were intravenously injected into sublethally (250rads) irradiated CD90.2 *nu/nu* BALB/c recipients. Donor derived progeny was assessed by staining spleen and peripheral lymph node preparations for CD90.1, CD4, CD8 and CD3ε and analysed by flow cytometry 6 weeks after transplantation. Values represent averaged frequencies of donor derived CD90.1+ cells ± standard deviation in injected (n) mice from 1 experiment. The DN1 CD45R population did not generate any progeny, whereas the DN1 CD117 population generated CD4-CD8-CD3ε+ cells.

*** $p < 0.001$

	Spleen		Peripheral Lymph Node	
	DN1 CD45R (n=8)	DN1 CD117 (n=2)	DN1 CD45R (n=8)	DN1 CD117 (n=2)
	Av. +/- S.D. (%)	Av. +/- S.D. (%)	Av. +/- S.D. (%)	Av. +/- S.D. (%)
CD4+CD3+	0.00	0.00	0.00	0.00
CD8+CD3+	0.00	0.00	0.00	0.00
CD4-CD8-CD3+	0.00	80.0 +/- 11.6 ***	0.00	83.1 +/- 6.6 ***

Discussion

The development of T-cells is dependent on the colonisation of the thymus by progenitor cells generated in the bone marrow of an adult individual. The process of colonisation is thought to be a gated phenomenon, with cells seeding the thymus in a wave-like fashion (Foss, Donskoy, et al. 2003). The most potent progenitor for T-cells has been identified with a surface phenotype of CD44+CD25-CD117+ (early thymic progenitor, ETP), and a robust functional potential to generate all T-lineage cells. However, the nature of this population remains unresolved. For instance, the ability to generate B-cells by this progenitor pool has been a subject of considerable debate. In the original study Allman *et al.* demonstrated a potential of the ETP to generate B-cells *in vivo*. Subsequently, studies have illustrated a lack of B-lineage potential *in vitro* (Balciunaite, Ceredig et al. 2005), as well as *in vivo* (Porritt, Rumfelt et al. 2004) in this population of progenitor cells. In addition, the study of Porritt *et al.* alluded to the existence of specific progenitor populations with discrete functional potentials, which seed the adult thymus. The latter line of thought is substantiated by a recent identification and characterisation of a committed T-cell precursor circulating in the adult blood (Krueger, von Boehmer 2007).

As detailed in previous chapters, we focused on the dissection of early double negative progenitor populations and identified a fraction of cells as yet uncharacterised. This population had the surface phenotype of DN1 cells and in addition expressed CD45R on the cell surface. There was a complete lack of CD117 and CD127 expression at the protein level, therefore this population is distinct from the early thymic progenitor and the common lymphoid progenitor-2 populations. At the genetic level, the DN1 CD45R population exhibited activity of T-cell specific genes, such as Notch-1, pre-Tα and GATA-3, and was completely positive for the fluorescent reporter, which would be

indicative of T-cell developmental potential. Therefore, the capacity of this thymocyte population to give rise to T-cells was examined.

A recently described *in vitro* culture system (Schmitt and Zuniga-Pflucker, 2002) which supports development of T-cells by providing a continuous Notch signal, was first employed to elucidate the functional potential of the DN1 CD45R population. In addition the DN1 CD117 positive (DN1 CD117) fraction of progenitor thymocytes was analysed in parallel and served as a positive control. In this system, where a Notch signal is continuously provided in combination with high levels of IL-7, SCF and Flt-3 ligand, the DN1 CD45R population generated T-cells with much faster kinetics than the canonical T-cell progenitor, the DN1 CD117 population. This would suggest that the former population is already committed to the T-lineage upon entering the thymus. It seems that the Notch signal is required for the DN1 CD45R population to generate T-cells, since culturing these cells on the OP9 bone marrow stroma alone did not yield any T-cell progeny, or even, B- and NK-cell progeny. This further underlines the idea of the DN1 CD45R population as being oligopotent and committed to the T-lineage.

In a more physiological environment, the foetal thymus, the DN1 CD45R population was able to transmigrate into the organ and generate progeny, thus illustrating the migratory potential associated with early T-cell progenitors and ultimately the developmental potential to generate T-cells. In this culture system, the transmigratory route is not physiological, since cells must enter through the thymic capsule rather than blood vessels. Nevertheless, the ability to go into the foetal thymus suggests that the adhesion requirements coupled with early stages of T-cell development are realised by the DN1 CD45R population. The DN1 CD45R population showed progression through the well-defined T-cell developmental checkpoints, illustrated by the generation of double positive cells, although T-lymphocytes generated by this fraction of cells are

short lived, seeing as the foetal thymic lobes were virtually empty after 14 days of culture. Noteworthy is the manner in which the DN1 CD117 population developed in this *in vitro* system. The generation of T-cells from the DN1 CD117 fraction was observed in distinct waves, with the first wave of T-cell generation coinciding with the wave of T-cell formation by the DN1 CD45R population. This would point to an existence of a T-cell committed precursor residing within the DN1 CD117 pool also.

To further understand the functionality of the DN1 CD45R population, *in vivo* transplantation experiments were carried out. The DN1 CD45R population revealed a potential to generate only T-cell progeny, however with a strong bias towards the CD8 T- or the CD8 NKT-cell phenotype. On very rare occasions CD4 T-cells were detected. The bias towards the CD8 lineage *in vivo* was unexpected, since both T-cell lineages were detected *in vitro*, furthermore, the DN1 CD45R fraction bears the CD4 co-receptor on the cell surface, thus an inverse predisposition might have been predicted. The life span of the different T-cell lineages could account for such an inclination. CD8 T-cells are longer lived than the CD4 lineage, and so this is in line with the observation that the CD4 T-cells were detected only after 4 weeks post-transplantation with 5000 cells, whereas the CD8 T-cells persisted until week 6. However, an earlier time point did not expose any donor-derived progeny from the DN1 CD45R population. In this case the experiments could have been limited by the small number of transplanted cells, since only 1000 cells were injected. In these experiments, chimerism was rare, and only CD8 T-cells were generated, thus further highlighting the limited functional potential of the DN1 CD45R population in this experimental system.

The DN1 CD117 fraction, on the other hand, showed a broad range of developmental potential, underlining the multipotent nature of this progenitor fraction at the population level. Foetal thymic organ cultures generated an array of haematopoietic lineages but

development of B-cells was never detected. This is not surprising however, given that the thymic environment is thought to be not largely permissive to the development of B-cells. Consequently, cultures on the OP9 bone marrow stromal monolayer revealed the B-cell potential of this population. In transplantation assays, all T-cell lineages were formed, even though preponderance towards the CD8 lineage was evident. The generation of B-cells was consistent. It has been shown that the frequency of B-cell progenitors in the DN1 CD117 fraction is 1 in 1000 cells in a four weeks old mouse (Ceredig R, Bosco N et al. 2007), yet even with 1000 injected cells B-lineage progeny was readily detectable. The ability of the DN1 CD117 population to generate myeloid progeny *in vitro* is well documented (Lee, Kim et al. 2001; Balciunaite, Ceredig et al. 2005), however no studies have addressed this issue *in vivo*. In these experiments, generation of myeloid progeny was observed, although with low frequency and low amount of donor derived cells. This can be attributed to competition of donor cells with the endogenous myeloid cells for developmental niches. The ability of the DN1 CD117 population to give rise to NK-cells is striking. It seems that this potential is first to be revealed because at 2 weeks post transplantation, the majority of donor derived cells are NK-cells. Experiments with 1000 donor cells also indicate that the ability to produce γ/δ T-cells precedes that of α/β T-cells, which could be explained by the possibility that NK-, as well as γ/δ T-cells, have fewer developmental checkpoints. In addition experiments with athymic hosts illustrate that γ/δ T-cells can be generated extrathymically.

Transplantation experiments revealed a difference between the *in vitro* and the *in vivo* developmental potential of DN1 CD45R population. Whereas *in vitro*, DN1 CD45R population generated CD4 and CD8 T-cells via a double positive intermediate, *in vivo*, donor derived progeny were mostly CD8 T-cells, and double positive cells were never

seen. This discrepancy could arise because the DN1 CD45R population is not a physiological T-cell progenitor. Alternatively, the inconsistency can be attributed to the manner in which the *in vivo* experiments were carried out. Cells were transplanted intravenously into *Rag2-/-cd132-/-* double mutants, which were sublethally irradiated. Irradiation would favour homing of progenitor cells to primary lymphoid organs, since the levels of chemoattractants, such as SDF-1, are elevated following irradiation (as reviewed by Lapidot, Dar et al. 2005). Furthermore, the hosts are alymphoid, therefore there is a complete absence of competitor cells. Taken together, this would point to a favourable environment for the cells to develop in. This, however, is clearly not the case, seeing as the progeny of DN1 CD45R cells are rarely detected in the thymus and the classical checkpoints of development, such as emergence of double positive cells, are never encountered. This could suggest extrathymic development of the DN1 CD45R population, yet experiments with athymic mice did not reveal any such event. Therefore it seems that the CD8 lineage cells that are generated by the DN1 CD45R population require a thymus. Thymic structure is severely compromised in mutants bearing dysfunctional RAG1/2 proteins (Hollander, Wang et al. 1995). This is reflected in a complete absence of the medulla and a distorted cortex, which in turn is mirrored in a disturbed composition of the DN1 pool of these mice (see Chapter 3). The DN1 CD45R population is virtually nonexistent in such mutants, in view of which it can be postulated that this progenitor population requires a fully functional thymus for proper development. Upon entering the thymus, the cells do not receive the required signals or receive inappropriate signals, either from the thymic stromal compartment or the more mature T-cell compartment, and are therefore unable to differentiate further. It is more likely that such signal emanates from the epithelial compartment of the thymus, seeing as an empty thymic lobe devoid of any mature T-cells, as is the case in FTOC, supports

the development of T-cells from the DN1 CD45R population. Notable is the fact that the inappropriate environment of the *Rag2-/-cd132-/-* double mutant is sufficient for the correct development of T-cells from the DN1 CD117 population, even though there is a prevalence towards the differentiation down the CD8 lineage. This indicates that there are distinct mechanisms which regulate the differentiation of T-cells from the two progenitor populations.

The ability of the DN1 CD117 cells to generate most haematopoietic lineages *in vitro* and *in vivo* would indicate that at the population level this fraction constitutes a multipotent progenitor. However, it does not preclude the possibility that at the single cell level the DN1 CD117 population is heterogeneous, consisting of lineage committed progenitors, which ultimately give rise to the array of haematopoietic cells seen here. One such example of a lineage committed progenitor seeding the thymus could be the DN1 CD45R population. This is a distinct population, separate from the canonical T-cell progenitor, which could contribute to the generation of the mature T-cell pool, either under steady state or during a pathological event.

Chapter 7

Final Discussion

The process of haematopoiesis originates from a common precursor, the haematopoietic stem cell (HSC). These cells are able to form all blood cell lineages through intermediate stages in the bone marrow of an adult. An exception to this practice is the generation of T-lymphocytes, which requires a specialised microenvironment encapsulated by the thymus. This, therefore, requires the migration of bone marrow derived progenitors to the thymus, via the blood. The prevailing model of haematopoiesis hypothesises that lymphoid and myeloid lineages diverge early on in the bone marrow, and a lymphoid committed progenitor cell, known as the common lymphoid progenitor (CLP), contributes to thymic ontogenesis (Kondo, Wessman et al. 1997). Functionally, these cells generate only lymphoid lineage progeny *in vivo* (Kondo, Wessman et al.1997), which is reflected by the transcriptional signature of these progenitors. Specifically, the CLP population transcribes genes only relevant to the lymphoid lineage (Akashi, He et al. 2003), this would therefore predict that early T-cell progenitors in the thymus are functionally limited to the lymphoid lineage and transcribe elements related only to that lineage.

The bone marrow harbours additional cells, which have been shown to have a T-cell developmental bias as compared to B- or myeloid cell generation. These cells were termed the early lymphoid progenitors (ELP) and were isolated based on expression of a fluorescent protein, reporting the expression of the endogenous *Rag-1* locus (Igarashi, Gregory at al. 2002). Additional surface markers defined these cells as $CD117^{high}$ and $Ly6A/E^{high}$, which positions them within a population harbouring all long-term haematopoietic stem and multipotent progenitor cells, thus suggesting that T-cell specification can occur as early as the multipotent progenitor stage.

The relationship between the bone marrow progenitor cells and the earliest progenitor cells in the thymus has not been directly established. It is still unclear, whether it is the

CLP or the ELP, which contributes to thymic ontogenesis. Early thymocyte differentiation is distinguished by the cell surface expression of CD44 and CD25, and the earliest stage is characterised by the high levels of CD44 expression and complete absence of CD25 (Lesley, Hyman et al. 1985; Ceredig, Lowenthal et al. 1985). These cells are called double negative 1 (DN1) and, in addition, the most robust T-cell differentiation potential resides in the fraction of cells that express the receptor tyrosine kinase c-kit (CD117). These cells have been branded the early thymic progenitors (ETP, DN1 CD117) and are therefore believed to be the direct precursors of DN2 and consequently DN3 thymocytes (Allman, Sambandam et al. 2003). Previously published reports and data presented here illustrate that the DN1 CD117 population is multipotent at the population level. These cells generated α/β and γ/δ T-cells, natural killer (NK) cells and dendritic cells (DC) in foetal thymic organ cultures, could develop B-cells on stromal monolayer co-cultures and, upon intravenous transfer, could even generate myeloid lineage cells, in addition to all other cell types derived *in vitro*. These developmental potentials were reflected in the genes that were transcribed within the DN1 CD117 population. A genome wide screen revealed that genes associated with a multitude of haematopoietic lineages were represented at the transcriptional level. Gene expression of transcription factors associated with B-cells, NK-cells, and even myeloid cells were detected. These included Sox-4, which is required for B-cell development (Schilham, Oosterwegel et al. 1996), Id-2, which is necessary for NK development (Yokota, Mansouri et al. 1999) and PU.1, which is indispensable for the development of myeloid cells (McKercher, Torbett et al. 1996). Additionally, transcripts of cell surface receptor genes, which induce differentiation of various haematopoietic lineages, were detected amongst the DN1 CD117 population, and included the macrophage colony stimulating factor 1 receptor and the erythropoietin receptor. At this point expression of

T-cell specific genes was already detected, consequently illustrating that the functional attributes of the DN1 CD117 population were reflected in the molecular signature of these cells. Interestingly, elements which inhibit generation of non-T lineages, were also expressed. One such example is GATA-2. This transcription factor has been shown to inhibit myelopoiesis as a direct result of Notch-1 receptor signalling (de Pooter, Schmitt et al. 2006). Notch-1 receptor signalling is essential in generating the DN1 CD117 population (Sambandam, Maillard et al. 2005), and the evidence of active signalling is illustrated by the expression of Notch-1 target genes, such as Hes-1, Dtx-1 and pre-Tα. This exemplifies the dual role of Notch-1 signalling in early T-cell progenitors, a direct role, where signals induce transcription of T-cell specific genes and an indirect role, where inhibition of alternative cell fates favours T-cell development. This, therefore, raises a question, why is myelopoiesis still evident in the DN1 CD117 population, albeit at very low frequency, if inhibitors of this lineage are expressed? One reason could be that the amount of GATA-2 at the protein level is not sufficient to block myeloid development. Another reason could be that GATA-2 is not expressed in every DN1 CD117 cell, therefore, those cells which do not express GATA-2 can differentiate down the myeloid pathway or alternatively, transcription of GATA-2 at this stage predisposes the cell to a complete inability to generate myeloid cells at the subsequent developmental checkpoint. Finally, the prohibitory effect of GATA-2 can be reversed by the adequate microenvironmental stimulus, therefore redirecting the cell to the myeloid lineage.

Upregulation of CD25 on the cell surface accompanies developing thymocytes to the next developmental stage, the double negative 2 (DN2) stage. The DN2 cells become more restricted in their developmental potential and loose the capacity to generate B- and myeloid cells, whilst retaining the ability to differentiate into NK- and clearly

T-cells (Schmitt, Ciofani et al. 2004). The genetic program of DN2 cells also becomes filtered. Expression of B- and myeloid lineage genes is either completely lost or significantly downregulated. In addition, expression of Id-2 is downmodulated, which reflects a decrease in the ability to generate NK-cells (Schmitt, Ciofani et al. 2004). Downregulation of Id-2 and the decreased ability to generate NK-cells could be a consequence of Notch-1 signalling, since it has been shown that DN2 cells, which are not exposed to high concentrations of Notch ligands, namely Delta like 1, generate more NK-cells than those DN2 cells, which encountered Notch ligands (Schmitt, Ciofani et al. 2004). Since signalling through the Notch-1 receptor has increased in DN2 cells as compared to DN1 CD117 cells, it can be postulated that at this developmental stage, Notch-1 signalling has a similar role in promoting T-cell differentiation in a positive fashion, by inducing T-cell specific genes and indirectly by inhibiting alternative fates. Alternatively, downregulation of Id-2 at the population level could be a result of the decreased frequency of Id-2 gene expression at the single cell level.

It has been suggested that Notch-1 signalling regulates expression of the receptor tyrosine kinase c-kit (CD117) in a positive manner (Massa, Balciunaite et al. 2006). This, however, is inconsistent with the patterns of CD117 and Notch-1 expression observed here. During development from DN1 CD117, where expression of CD117 is high on the cell surface, to DN2, CD117 is downregulated. This is paralleled by an increase in Notch-1 gene expression and successive upregulation of Notch-1 target genes. Upon further development along the T-cell axis to the DN3 stage, Notch-1 and its target genes are further upmodulated, whereas expression of CD117 is downregulated at the transcript and protein levels. This suggests that Notch-1 signalling negatively regulates expression of CD117. This inconsistency in observations could be due to the source of cells used in the study of Massa *et al.* These authors employed

Pax5 deficient pro-B cells in their experiments, which might have different regulatory circuits in place, thus yielding a different outcome.

Downregulation of CD44 marks the DN3 stage of T-cell development. DN3 cells are only able to differentiate along the T-cell axis, generating α/β or γ/δ T-cells only. This is mirrored in a further filtration of the genetic program active in these cells. T-lineage genes are dramatically upregulated, whereas expression of genes associated with alternative fates are diminished. This presents a scenario of early T-cell development, where gene expression patterns reflect the functional properties of progenitor thymocytes (Figure 7.1). Upregulation of T-cell genetic program is paralleled by an increased commitment to generate T-cells, which replaces alternative genetic programs and ceases respective fates. Interestingly, alternative genetic programs are not fully eradicated, since overexpression of myeloid related transcription factors, PU.1 and C/EBPα, in DN3 cells reprograms these cells into macrophages and dendritic cells (Laiosa, Stadtfeld et al. 2006). This suggests that even at the DN3 stage, some loci which contain elements necessary for myeloid or DC development are still accessible to the actions of PU.1 and C/EBPα. It would be interesting to see the progression of DN3 differentiation into macrophages and DCs in this system, do the DN3 cells dedifferentiate and therefore revert to a transcriptional fingerprint of DN2 and DN1 cells, or do these cells directly transdifferentiate into macrophages and DCs?

Regulation of genetic programs at the population level could be the direct consequence of regulating the gene expression of respective loci. Alternatively, the difference in patterns of gene expression seen between the early stages of conventional T-cell development could arise due to the alterations in population composition (Figure 7.2). Expression of a variety of genetic programs in the DN1 CD117 population could be a reflection of heterogeneity of this population. The DN1 CD117 population could

contain specific progenitor subsets, which possess distinct developmental attributes and thus express different genetic programs. Some evidence of functional heterogeneity in the DN1 population has been illustrated (Porritt, Rumfelt et al. 2004). These authors showed that the potential to generate B- and NK-cells diverged between different subsets of DN1 CD117+ cells. Noteworthy is the observation that cells capable of B-cell development expressed slightly lower levels of CD117, therefore were not strictly of a DN1 $CD117^{high}$ (ETP) phenotype. At the next developmental stage, the DN2 stage, population composition becomes more homogeneous, only some cells were able to progress to the next stage, therefore upregulation of T-cell genes is a result of more frequent T-cell progenitors within the whole DN2 population. Downregulation of alternative genetic programs reflects the less frequent encounter with progenitor cells specifying alternative fates. Finally, at the DN3 stage, only T-cell progenitors remain, therefore a T-cell specific genetic program is seen (Figure 7.2).

Figure 7.1 Developmental Progression During Early Thymic Ontogenesis I.

A model of T-cell differentiation illustrating a sequential loss of alternative lineages as a result of a filtration of genetic programs. The DN1 CD117 population is multipotent, transcribing elements associated with multiple haematopoietic lineages and therefore generating a diverse repertoire of haematopoietic cells. GATA-2 is expressed and negatively regulates myeloid development, but the protein levels are not sufficient to fully abort this differentiation event. Upon progression to the DN2 population, genes representing alternative lineages are downmodulated, thus the functional outcome is a limited differentiation potential. Inhibition of alternative lineages could be a direct result of the T-cell specific genetic program, such as signalling through the Notch-1 receptor. At the DN3 stage, only T-cells are generated and therefore the T-cell genetic program is vastly predominant. Upregulation of transcription factors such as Sox5 and T-box6 might lock the T-cell identity of this population.

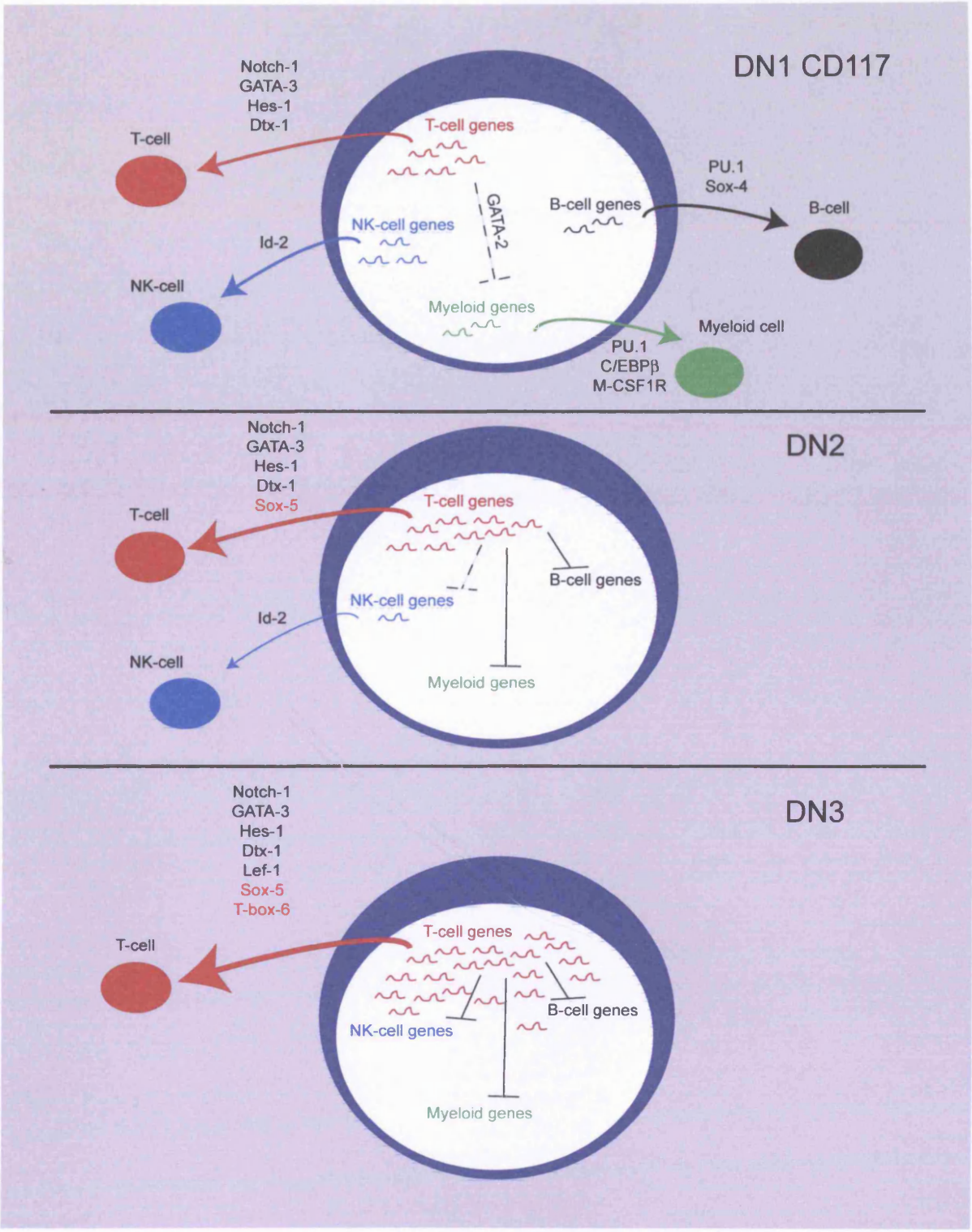
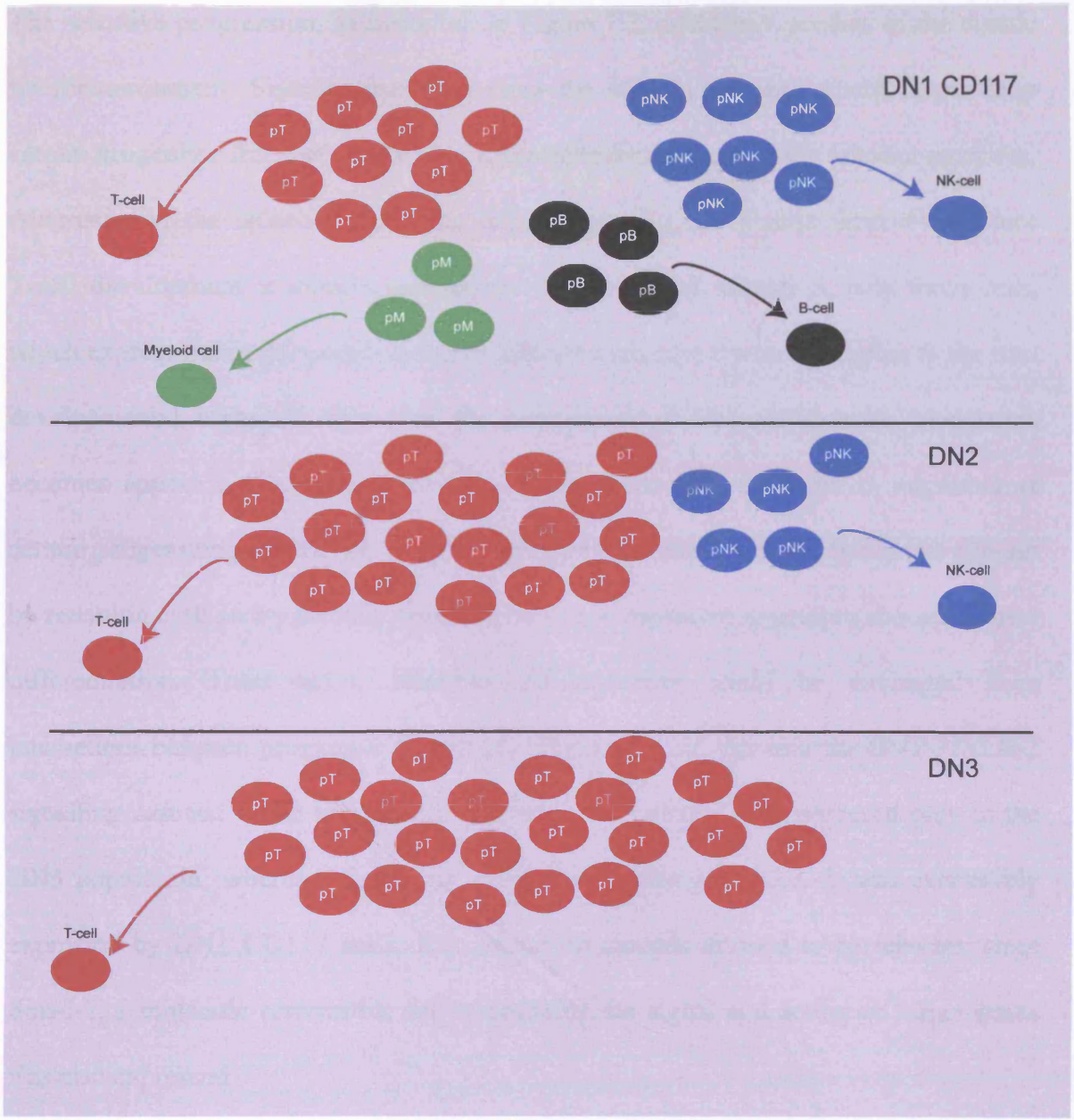

DN1 CD117
Notch-1
GATA-3
Hes-1
Dtx-1
T-cell
T-cell genes
PU.1
Sox-4
B-cell genes
B-cell
NK-cell genes
GATA-2
Id-2
NK-cell
Myeloid genes
Myeloid cell
PU.1
C/EBPβ
M-CSF1R
DN2
Notch-1
GATA-3
Hes-1
Dtx-1
Sox-5
T-cell
T-cell genes
B-cell genes
NK-cell genes
Id-2
NK-cell
Myeloid genes
DN3
Notch-1
GATA-3
Hes-1
Dtx-1
Lef-1
Sox-5
T-box-6
T-cell
T-cell genes
B-cell genes
NK-cell genes
Myeloid genes

Figure 7.2 Developmental Progression During Early Thymic Ontogenesis II.

A model of early T-cell differentiation illustrating distinct origins of differentiation products in double negative populations. The DN1 CD117 population is heterogeneous, harbouring distinct lineage committed progenitor subsets, which express lineage specific genetic programs. The next developmental stage, the DN2 population is more homogeneous, containing only certain lineage committed progenitor subsets and therefore the genetic program is more refined. Finally, the DN3 population constitutes only T-cell progenitors, thus the T-cell specific genetic program prevails.


DN1 CD117
pT
pNK
pB
pM
T-cell
NK-cell
B-cell
Myeloid cell
DN2
T-cell
NK-cell
DN3
T-cell

The selective progression, as described in Figure 7.2, could be a product of the thymic microenvironment. Signals emanating from the stromal elements could favour only certain progenitor fractions, since only some cells would express the relevant receptors. Alternatively, the selection could be intrinsic to progenitor cells themselves. Since T-cell development is closely coupled to the process of migration, only those cells, which express particular combination of adhesion receptors, would progress to the next developmental stage. In this case the importance of integrin receptor modulation becomes apparent. The integrin receptors would not only facilitate in migration of certain progenitor populations, thereby assisting their development, but could also act by retaining cells in a particular location, therefore inhibiting migration and subsequent differentiation. Then again, selective differentiation could be envisaged from interactions between progenitor thymocytes themselves. In this case the BMP-7/ALK-2 signalling cascade could play a role. Expression of BMP-7 was observed only in the DN3 population, whereas, the receptor for this morphogen, ALK-2, was exclusively expressed by DN1 CD117 cells. This signalling cascade seemed to be relevant, since Smad-1, a molecule responsible for transmitting the signal and acting on target genes was also expressed.

Single cell multiplex PCR analysis, as well as single cell functional studies would help to address whether early thymic progenitors are truly multipotent or indeed contain separate lineage committed progenitor fractions. Some recent experimental evidence has demonstrated that rare cells within the DN1 CD117 population harbour the capacity to generate T-, B- and dendritic cells on the single cell level (Benz and Bleul 2005). Generation of natural killer cells was not detected in cultures where T-, B- and dendritic cells were generated, therefore illustrating that these cells were not truly multipotent, some lineage restriction already took place. Additionally, a recent report identifying

committed T-lineage precursors in adult circulation would suggest that commitment to the T-cell lineage can occur pre-thymically and so a fraction of early thymic progenitors would constitute already committed T-lineage progenitor cells (Krueger and von Boehmer 2007). Since commitment to the T-cell lineage coincides with human CD2::iCre driven fluorescent reporter, it was interesting to note that a small fraction of DN1 CD117 cells were already completely positive for the reporter. This would suggest that, indeed, a fraction of DN1 CD117 cells constitutes a committed T-lineage progenitor. No concrete evidence has so far been generated to support this idea, but an observation that T-cell development takes place in distinct waves in foetal thymic organ cultures, alludes to a possibility that generation of T-cell progeny with a faster kinetic is a consequence of contribution by a T-lineage committed progenitor.

It was surprising to see that the reporter system used in this study completely marked a fraction of DN1 cells, which did not express CD117. It is currently believed that DN1 CD117 negative cells do not contribute to T-cell development (Allman, Sambandam et al. 2003). Although one study illustrated that T-cell development can proceed from DN1 CD117 negative cells *in vitro* (Porritt, Rumfelt et al. 2004). However, both studies overlooked the population of cells characterised here, since depletion strategies involved in those experiments included the CD45R antibody in the depletion cocktail. Here a population of DN1 CD117 negative cells was isolated and characterised. The hallmark of this population was its cell surface expression of CD45R. A population with a similar phenotype has been identified in the bone marrow (Martin, Aifantis et al. 2003). This population was termed the common lymphoid progenitor 2 (CLP-2) and was isolated based on expression of the human CD25 driven by a putative pre-Tα promoter in a transgenic mouse line. These cells expressed intermediate levels of CD117 and expressed the interleukin 7 receptor α chain (CD127).

This phenotype is inconsistent with the one observed on the DN1 CD45R population described here. The DN1 CD45R cells were completely negative for CD117 and CD127. In addition, this population was found in circulation in parallel with the CLP-2 population (Krueger and von Boehmer 2007), suggesting a distinction between these cells.

The DN1 CD45R population exhibited complete expression of the human CD2::iCre driven fluorescent reporter and on the molecular level expressed elements associated with the T-cell lineage. These elements included Notch-1 gene expression, seen to the comparable level as the DN1 CD117 population, and even higher gene expression levels of pre-Tα and Rag-1 than the DN1 CD117 population. Absence of the primary Notch-1 target genes but high expression of pre-Tα would suggest that the Notch signal was received already some time ago, possibly pre-thymically. Taken together, this would point that the DN1 CD45R population harbours T-lineage developmental potential and therefore constitutes a T-cell lineage committed precursor.

The DN1 CD45R population exhibited a robust ability to generate only T-cell progeny *in vitro*. Differentiation of the DN1 CD45R population was seen in co-culture experiments with OP9 bone marrow stromal cells expressing the Notch ligand Delta Like Ligand 1. Additionally, robust T-cell development was observed in foetal thymic organ cultures, which suggests that the DN1 CD45R population is able to transmigrate into the thymus, a property associated with early progenitor cells. In both cases the development of single positive T-cells was via a double positive intermediate, demonstrating that the DN1 CD45R population follows the well-defined stages of thymocyte differentiation. The kinetics of T-cell development from this DN1 fraction was striking. T-cell progeny derived from the DN1 CD45R population was detected much earlier than the progeny of DN1 CD117 cells on the OP9-Dll1 layer, suggesting

that the DN1 CD45R population is already committed to the T-cell lineage and is thus able to generate T-cells more quickly. In foetal thymic organ cultures the DN1 CD45R population also exhibited rapid kinetics of T-cell development, generating progeny faster than the mainstream wave derived from DN1 CD117 cells. Expression of the fluorescent reporter together with high levels of T-cell lineage genes and a robust and rapid potential to generate T-cells *in vitro* would propose that the DN1 CD45R population is a T-lineage committed cell.

The limited developmental potential of the DN1 CD45R population was further demonstrated by *in vivo* transplantation experiments. Upon intravenous transfer of purified progenitor cells into sublethally irradiated hosts only T-cell progeny was detected from DN1 CD45R donor cells. Remarkably, donor derived cells were predominantly CD8 T-cells. This points to the fact that the DN1 CD45R population is a T-lineage committed progenitor, which originates in the bone marrow and is distinct from the canonical DN1 CD117 progenitor population, therefore suggesting that generation of T-cells can occur via independent pathways.

The interpretation of the *in vivo* transplantation experiments is partially complicated by rare chimerism and low numbers of donor derived cells. This could be argued that the DN1 CD45R population is not a physiological T-cell progenitor. On the other hand, it should be noted that the DN1 CD45R population is quiescent, therefore recovery of donor derived cells was difficult. Another explanation could be the microenvironment of the host. The hosts used for transplantation experiments were mutants, which carried a double deficiency in the *Rag-1* and the *cd132* loci. The thymic architecture in mice carrying the *Rag-1* mutation exhibits abnormalities. This is paralleled by a severe disruption in composition of DN1 progenitor cells. In these mice the DN1 CD45R population is virtually absent and additionally, the DN1 CD117 population shows a

severe reduction in cell numbers. This suggests that signals derived from the stromal components of the thymus are insufficient to sustain survival of these populations. Alternatively, lack of any mature T-cell pools can influence the poor survival of DN1 progenitors. However, the latter argument is not easily reconciled with the observation that differentiation of the DN1 CD45R population in foetal thymic lobes devoid of any mature T-cells is observed. Although, the environment provided by the foetal and adult thymi are different, thus, the foetal thymic environment alone could be sufficient to support the differentiation of DN1 CD45R progenitors. Transplantation experiments with wild type hosts, which have an unaltered thymus, are currently underway to address the effect of thymic structure on DN1 progenitors. Noteworthy is the observation that DN1 CD117 progenitors progressed through classical stages of T-cell differentiation and generated all T-cell lineages in appreciable numbers in the mutant environment, suggesting that differentiation of DN1 CD117 and DN1 CD45R progenitor cells is governed by distinct sets of signals, thus further separating the two populations. Altogether, this exemplifies that during adult thymopoiesis, distinct progenitor populations can seed the thymus and contribute to the generation of mature T-cell lineages.

The nature of the bone marrow cells which ultimately contribute to T-cell generation is still unresolved. Studies have demonstrated that the earliest progenitor population in DN1 by surface phenotype resemble multipotent progenitors in the bone marrow (Sambandam, Maillard et al. 2005). These cells, termed the thymic seeding progenitors (TSP), express CD135, which is a marker of multipotent progenitors in the bone marrow, therefore suggesting that colonisation of the thymus is achieved by a bone marrow derived multipotent progenitor cell. A respective population in circulation has also been isolated (Schwarz and Bhandoola 2004). Our study has come to a similar

conclusion. Since the DN1 population capable of efficiently generating T-cells expresses CD117, a marker of early haematopoietic progenitor cells, generates a variety of haematopoietic lineages *in vivo* and additionally expresses genetic elements associated with a multitude of blood cell types, would suggest that, indeed, at the population level, the DN1 CD117 population constitutes a multipotent progenitor. A population with a similar phenotype was detected in the blood, thus providing a link between the DN1 CD117 and the multipotent progenitor cells in the bone marrow. It is unlikely that the common lymphoid progenitor population is a direct precursor to the DN1 CD117 population. The surface phenotype is quite different between these populations but the strongest evidence comes from the genetic analysis of the DN1 CD117 population. This population of cells exhibited transcripts associated with the myeloid lineage, whereas a previously published report promulgated that the common lymphoid progenitor is restricted to the expression of lymphoid lineage genes only (Akashi, He et al. 2003), specifically, the B-cell genetic program was predominant. This would therefore point to the origin of the DN1 CD117 population upstream of the CLP and the CLP as an intermediate stage in B-cell development, a heterogeneous population, which has some residual T-cell developmental potential.

In conclusion, we have confirmed the heterogeneity of the DN1 progenitor thymocyte population by careful and comprehensive flow cytometric analysis and additional illustration that the DN2 population is also heterogeneous, which was demonstrated with the help of a fluorescent reporter system. The fluorescent reporter system also aided in unequivocal demonstration that DN1 heterogeneity is a result of thymic colonisation by distinct progenitor populations, which could be traced to different origins in the bone marrow. Functional analysis of DN1 progenitors illustrated that thymic colonisation is achieved by cells with discrete functional attributes. The

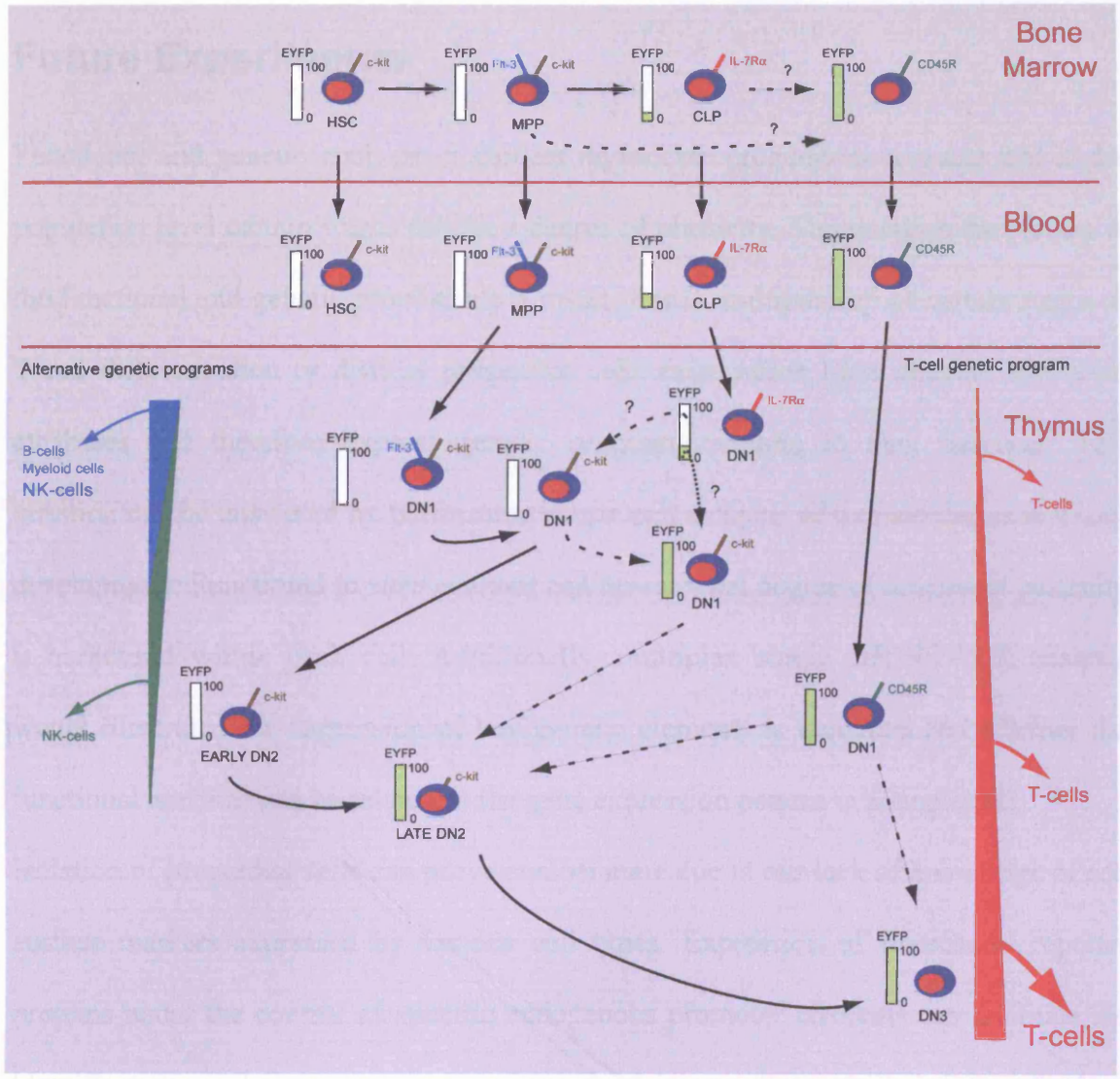
canonical T-cell developmental pathway stems from a multipotent DN1 CD117 population, which generates T-cells upon sequential loss of alternative lineage fates. In addition, T-lineage committed progenitors seed the thymus and contribute to thymic ontogenesis. The T-cell lineage committed precursor identified and characterised in this study can be defined as DN1 CD45R. The presence of two separate precursor populations in the adult thymus points to an existence of separate developmental pathways for T-cell differentiation, thus exemplifying the plasticity of the haematopoietic system. The relevance of each pathway to the contribution of T-cell generation during a pathological event remains to be understood.

In addition, we have here generated a sequence of molecular events that take place during specification and commitment to the T-cell lineage. Each developmental stage can now be identified and defined by a molecular signature. A combination of activated and silenced genes can now serve as a definition of cellular identity. Gene expression programs can serve to establish precursor-product relationships, where an overlap in patterns of gene expression can imply a direct relationship between populations. Genome wide analysis of additional cellular populations derived from the bone marrow, or even the thymus, would aid in construction of an ontological scheme of early lymphoid differentiation based on transcriptional signatures of the respective populations.

Figure 7.3 A Model of Early T-cell Development in the Adult Mouse.

Development of T-cells originates in the bone marrow of an adult. Bone marrow derived progenitor cells seed the thymus via the blood. Thymic colonisation can be achieved by separate progenitor populations, which can contribute to discrete stages of T-cell development. The multipotent progenitor population from the bone marrow is found in circulation and is the most likely precursor to the DN1 CD117 population in the thymus. The common lymphoid progenitor is also found in circulation and an analogue to the common lymphoid progenitor is detected in the thymus. The contribution of this progenitor fraction to T-cell development is unclear.

An additional progenitor population was identified in the thymus, which is distinct from the canonical DN1 CD117 progenitor population. These cells express CD45R on the cell surface and can be traced to the bone marrow. The DN1 CD45R progenitor population exhibits the characteristics of a committed T-cell progenitor, which therefore suggests that commitment to the T-cell lineage can take place in the bone marrow.


Bone Marrow
Blood
EYFP
100
0
c-kit
HSC
Flt-3
MPP
IL-7Rα
CLP
CD45R
?
Alternative genetic programs
T-cell genetic program
B-cells
Myeloid cells
NK-cells
DN1
Thymus
T-cells
EARLY DN2
LATE DN2
DN3

Future Experiments

Functional and genetic analysis of earliest thymocyte progenitors revealed that at the population level certain stages exhibit a degree of plasticity. The question then arises, is the functional and genetic promiscuity a result of true multipotency of certain stages of T-cell differentiation or distinct progenitor cells exist which have discrete functional attributes and therefore express genetic programs relating to their function? This question can be answered by performing single cell analysis of defined stages in T-cell development. Functional *in vitro* analysis can reveal what degree of functional plasticity is harboured within each cell. Additionally, multiplex single cell RT-PCR analysis would illustrate how expression of key genetic elements is regulated and whether the functional attribute can be related to the gene expression pattern in a single cell.

Isolation of progenitor cells can prove cumbersome due to our lack of knowledge of cell surface markers expressed by various cell types. Expression of fluorescent reporter proteins under the control of specific endogenous promoter elements can facilitate the identification, isolation and characterisation of intermediate stages of lymphoid or myeloid development to further help in understanding the process of haematopoiesis, for example, where does T-cell fate specification take place and what are the intermediate stages involved in the bone marrow? Combination of endogenous reporters with systems to permanently mark cells by a fluorescent protein of different colour can facilitate in pin pointing the exact intermediate stages involved. Efforts are currently under way in our laboratory to generate models, which will help us answer these questions.

The fluorescent reporter system utilised in this study demonstrated heterogeneity of DN2 cells. It would now be interesting to check, whether this reporter system can

facilitate the identification of the point of divergence between natural killer cells and T-cells amongst this population. A prediction would be that upon expression of the fluorescent label, the potential to generate natural killer cells would diminish. Both functional and transcriptional analysis of defined populations and single cells within these populations would allow us to answer this question.

Finally, the genome wide transcriptional analysis of progenitor thymocytes has generated a transcriptional definition of each progenitor stage. Analysis of transcriptional signatures from isolated populations in the bone marrow, the ELP, or the thymus, the DN1 CD45R, would allow to align these populations with the already defined stages and thus construct an ontological scheme of early lymphoid or T-cell differentiation, based on the gene expression signatures of each progenitor population.

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